USER GUIDE

gibco® by life technologies™

Freedom™ DG44 Kit

For transfection of CHO DG44 Cells (cGMP banked) and development of stable cell lines for protein production

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Product information

Kit contents and storage

Introduction

The components of the Freedom™ DG44 Kit and their shipping and storage conditions are listed below.

Item	Amount	Shipping	Storage
CHO DG44 Cells (cGMP banked), 1×10^7 cells/mL	1 mL	Dry ice	Liquid nitrogen
CD DG44 Chemically Defined Medium	1000 mL	Ambient temperature	2°C to 8°C, **protect from light**
CD OptiCHO™ Medium	1000 mL	Ambient temperature	2°C to 8°C, **protect from light**
OptiPRO™ SFM	100 mL	Ambient temperature	2°C to 8°C, **protect from light**
FreeStyle™ MAX Reagent	1 mL	Wet ice	2°C to 8°C, **do not freeze**
Pluronic® F-68, 10%	100 mL	Ambient temperature	15°C to 30°C
L-glutamine, 200 mM	100 mL	Dry ice	–20°C to –5°C, **protect from light**
Geneticin® Selective Antibiotic, 50 mg/mL	100 mL	Ambient temperature	2°C to 8°C, **protect from light**
One Shot® TOP10 Chemically Competent *E. coli* (20 reactions)	21 × 50 µL	Dry ice	–80°C
pcDNA™3.3-TOPO® TA Cloning Kit	1 kit	Dry ice	–20°C (reagents), –80°C (competent cells)
pOptiVEC™-TOPO® TA Cloning Kit	1 kit	Dry ice	–20°C (reagents), –80°C (competent cells)
Flash drive (contains the user manual for the Freedom™ DG44 Kit)	1 flash drive	Ambient temperature	Room temperature

Description of the system

Freedom™ DG44 Kit

The Freedom™ DG44 Kit is designed for easy cloning and expression of recombinant proteins in dihydrofolate reductase (DHFR)-deficient, Chinese hamster ovary (CHO)-derived CHO DG44 Cells (cGMP banked) in suspension culture. The Freedom™ DG44 Kit provides reagents for:

- cloning the gene(s) that encode a single or two-subunit protein
- transfecting the DNA constructs into CHO DG44 Cells (cGMP banked) with high efficiency
- generating stable cell lines that produce your protein of interest

Components of the Freedom™ DG44 Kit

- **pOptiVEC™-TOPO® TA Cloning Kit:** A TOPO®-adapted bicistronic plasmid and reagents for cloning a PCR product containing a mammalian secretion signal and each subunit, separately, of your protein of interest. See the next page for more information.
- **pcDNA™3.3-TOPO® TA Cloning Kit:** A TOPO®-adapted plasmid and reagents for cloning a PCR product containing a mammalian secretion signal and each subunit, separately, of your protein of interest. See the next page for more information.
- **CHO DG44 Cells (cGMP banked):** cGMP banked, DHFR-negative, CHO-derived cells adapted to high density, serum-free suspension culture in CD DG44 Medium that are capable of producing high levels of secreted, recombinant protein. See page 10 for more information.
- **CD DG44 Medium:** Defined, serum-free medium supplemented with hypoxanthine and thymidine to allow growth of DHFR-negative CHO DG44 Cells (cGMP banked). See page 11 for more information.
- **FreeStyle™ MAX Reagent:** A proprietary, animal origin-free formulation for high transfection efficiency of plasmid DNA into CHO DG44 Cells (cGMP banked). See page 12 for more information.
- **CD OptiCHO™ Medium:** Defined, serum-free medium formulated for selection and growth of CHO DG44 Cells (cGMP banked) expressing DHFR and the recombinant protein of interest. See page 13 for more information.
- **Additional components:** OptiPRO™ SFM (serum-free medium) for optimal DNA:lipid complex formulation, L-glutamine for increased media stability, Geneticin® Selective Antibiotic for stable cell line selection, and the surfactant Pluronic® F-68 to control shear forces in suspension culture.

Advantages of the Freedom™ DG44 Kit

The Freedom™ DG44 Kit provides the following advantages for protein production in mammalian cells:

- DHFR-deficient cGMP banked CHO DG44 Cells derived from CHO cells (Urlaub *et al.*, 1983; Urlaub *et al.*, 1986) provide stable and accurate glycosylation (Sheeley *et al.*, 1997; Werner *et al.*, 1998) and are observed to yield accurate glycoproteins.
- FreeStyle™ MAX Reagent offers high transfection efficiency of suspension CHO cells with low cytotoxicity.
- FreeStyle™ MAX Reagent, CD DG44 Medium and CD OptiCHO™ Medium are animal origin-free and serum-free.
- The kit allows for stable cell-line development, with all steps using serum-free conditions and animal origin-free (AOF) components.

TOPO® TA Cloning Vector Kits

The Freedom™ DG44 Kit contains the pOptiVEC™-TOPO® TA Cloning Kit and the pcDNA™3.3-TOPO® TA Cloning Kit (also available separately, see page *5* for details).

- The pOptiVEC™-TOPO® TA Cloning Kit contains the pOptiVEC™-TOPO® vector, a TOPO®-adapted bicistronic plasmid that allows rapid cloning of a PCR product containing a mammalian secretion signal and the gene of interest downstream of the CMV promoter. In the pOptiVEC™-TOPO® vector, the transcription of the gene of interest is separated from the dihydrofolate reductase (DHFR) auxotrophic selection marker by an internal ribosome entry site (IRES), allowing transcription of the gene of interest and the selection marker on the same mRNA.
- The pcDNA™3.3-TOPO® TA Cloning Kit contains the pcDNA™3.3-TOPO® vector, a TOPO®-adapted plasmid that allows rapid cloning of a PCR product containing a mammalian secretion signal and the gene of interest downstream of the CMV promoter. The pcDNA™3.3-TOPO® contains a neomycin resistance gene, allowing selection using Geneticin® Selective Antibiotic.

Continued on next page

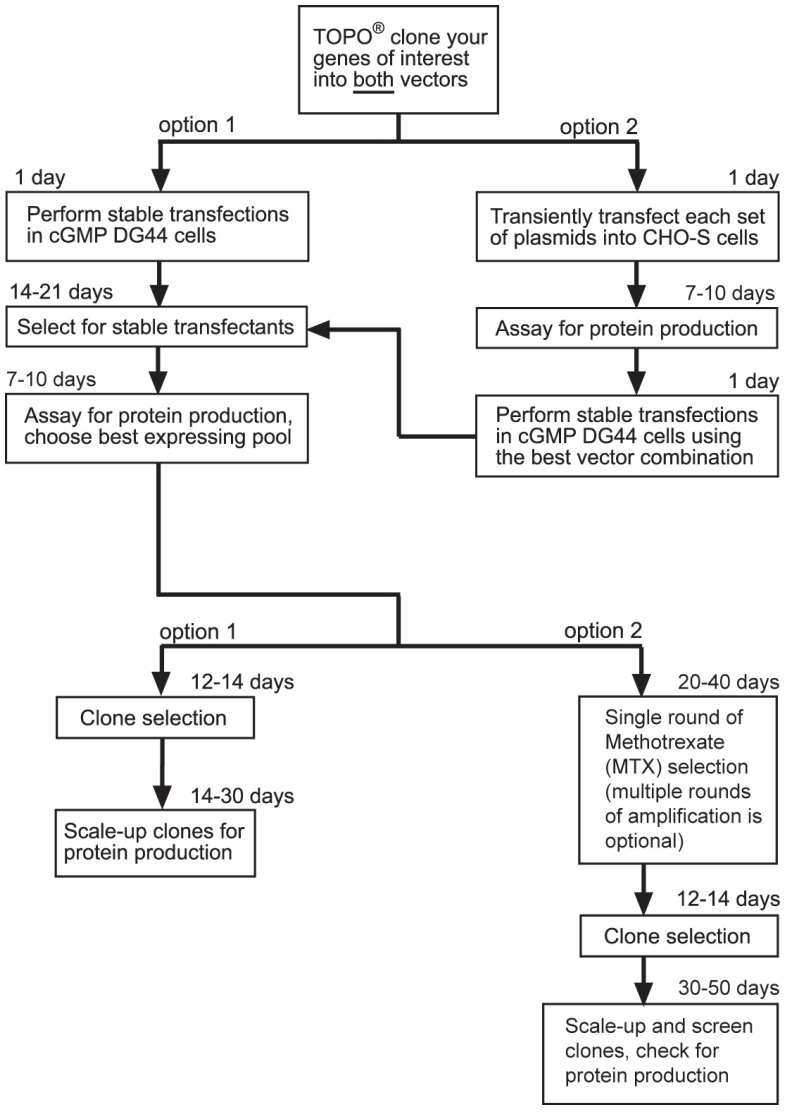
Experimental flowchart for two-subunit protein expression

Introduction

The diagram below schematically depicts the steps necessary to express your two-subunit protein of interest using the Freedom™ DG44 Kit, and it shows several common pathways from stable transfectants to clone scale-up. **Note that the times shown for various experimental steps are approximations, and the actual times depend on your protein of interest and the specific workflows you choose.**

See **Methods for Two-subunit Protein Expression**, pages 20–41 for detailed protocols.

Note: For detailed information on cloning each subunit of your protein of interest into pOptiVEC™-TOPO® TA and pcDNA™3.3-TOPO® TA vectors, refer to the pOptiVEC™-TOPO® TA Cloning Kit and pcDNA™3.3-TOPO® TA Cloning Kit manuals (part nos. 25-0977 and 25-1010, respectively) that are available at **www.lifetechnologies.com/manuals**.


TOPO® clone your genes of interest into both vectors
option 1
option 2
1 day
Perform stable transfections in cGMP DG44 cells
1 day
Transiently transfect each set of plasmids into CHO-S cells
14-21 days
Select for stable transfectants
7-10 days
Assay for protein production
7-10 days
Assay for protein production, choose best expressing pool
1 day
Perform stable transfections in cGMP DG44 cells using the best vector combination
option 1
option 2
12-14 days
Clone selection
14-30 days
Scale-up clones for protein production
20-40 days
Single round of Methotrexate (MTX) selection (multiple rounds of amplification is optional)
12-14 days
Clone selection
30-50 days
Scale-up and screen clones, check for protein production

Experimental flowchart for single-subunit protein expression

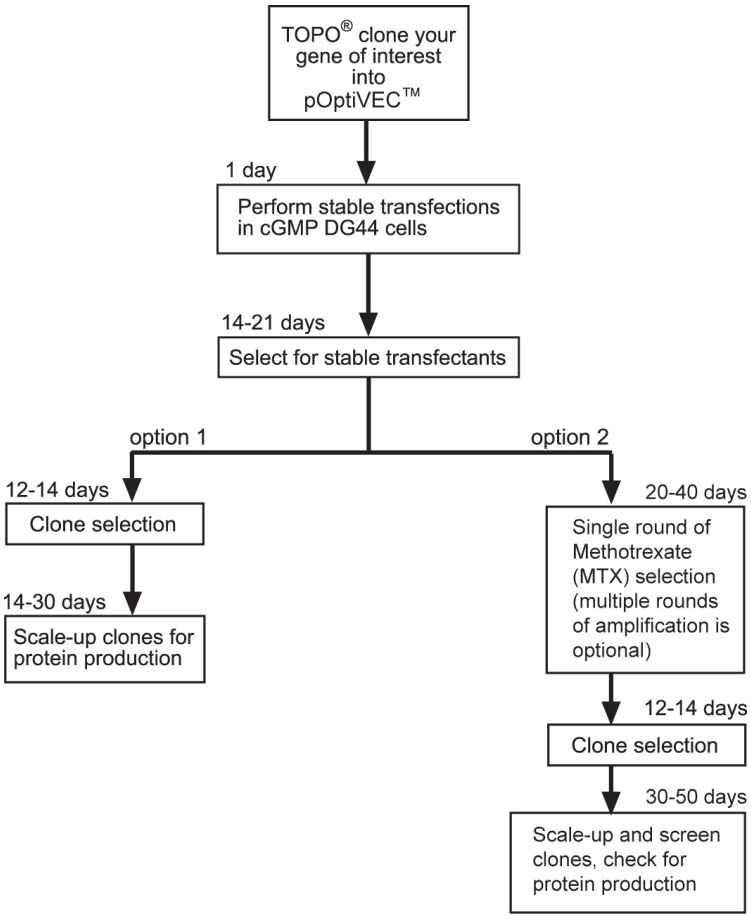
Experimental flowchart for single-subunit protein expression

The diagram below schematically depicts the steps necessary to express your single-subunit protein of interest using the Freedom™ DG44 Kit as well as several common pathways from stable transfectants to clone scale-up. **Note that the times shown for various experimental steps are approximations, and the actual times will depend on your protein of interest and the specific workflows you choose.**

See **Methods for Single-subunit Protein Expression**, pages 42–61, for detailed protocols.

Note: For detailed information on cloning your protein of interest into the pOptiVEC™-TOPO® TA vector, refer to the pOptiVEC™-TOPO® TA Cloning Kit manual (part no. 25-0977), available at **www.lifetechnologies.com/manuals**.

Important: If you are expressing a single-subunit protein, you must generate your expression construct using the pOptiVEC™-TOPO® TA vector. The pOptiVEC™ expression construct allows genomic amplification by MTX amplification.



CHO DG44 cells (cGMP banked)

Introduction

The CHO DG44 cell line is a dihydrofolate reductase (DHFR)-deficient cell line derived from suspension Chinese hamster ovary (CHO) cells (Urlaub *et al.*, 1983; Urlaub *et al.*, 1986). CHO DG44 Cells (cGMP banked), manufactured under cGMP guidelines, are adapted to suspension culture in CD DG44 Medium. Frozen cells are supplied at a concentration of 1×10^7 cells/mL and they may be thawed directly into CD DG44 Medium (see **Thawing and subculturing CHO DG44 Cells**, page 15).

Parental cell line

The CHO cell line is a stable aneuploidy cell line established from the ovary of an adult Chinese hamster (Puck *et al.*, 1958). CHO cells are commonly used cell lines for transfection, expression, and large-scale production of recombinant proteins.

DHFR

DHFR catalyzes the reduction of 5, 6-dihydrofolate to 5, 6, 7, 8-tetrahydrofolate, which is essential for DNA synthesis. CHO-derived DG44 Cells (cGMP banked) lack DHFR activity and they must be propagated in medium containing the purine precursors hypoxanthine and thymidine (HT), unless the cells are stably transfected with a vector that expresses DHFR.

DHFR also functions as a genomic amplification marker for your gene of interest using methotrexate (MTX) selection (Kaufman *et al.*, 1985; Tanaka *et al.*, 2002). See page 35 for more details on genomic amplification using MTX.

Characteristics of CHO DG44 cells (cGMP banked)

The CHO DG44 cell line:

- is cGMP banked with documentation and license available for commercial use
- is adapted to serum-free suspension growth in CD DG44 Medium containing hypoxanthine and thymidine
- demonstrates high transfection efficiencies with FreeStyle™ MAX Reagent
- allows suspension cultures to be transfected in CD DG44 Medium

CAUTION!

As with other mammalian cell lines, when working with CHO DG44 Cells (cGMP banked), handle them as potentially biohazardous material under at least Biosafety Level 1 containment.

Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

CD DG44 Medium

Introduction

CD DG44 Medium is a defined, serum-free medium containing hypoxanthine and thymidine for high-density suspension culture of untransfected CHO DG44 Cells. The medium contains no human or animal origin components.

Features of CD DG44 Medium

CD DG44 Medium is:

- chemically defined, containing no proteins or peptide components of animal, plant, or synthetic origin, and no undefined hydrolysates or lysates
- supplemented with hypoxanthine and thymidine (HT) for growth of DHFR-negative cells
- formulated without L-glutamine to avoid problems associated with L-glutamine degradation, including ammonia accumulation
- formulated without Pluronic® F-68
- formulated without phenol red to minimize potential estrogen-like effects

Preparing Complete CD DG44 Medium

- CD DG44 Medium requires supplementation with L-glutamine. Aseptically add L-glutamine to a final concentration of 8 mM to the medium before use.
- CD DG44 Medium requires the addition of a surfactant to protect against shear forces in suspension culture. Aseptically add 18 mL/L of Pluronic® F-68 to the medium before use.
- **Store complete CD DG44 Medium at 2°C to 8°C, protected from light.**

Growth characteristics of CHO DG44 Cells (cGMP banked) in CD DG44 Medium

Typically, CHO DG44 Cells (cGMP banked) cultured in CD DG44 Medium have a doubling time in the range of 22–26 hours (doubling time can exceed 26 hours during the first few passages after the cells have been thawed.)

Do not allow CHO DG44 Cells (cGMP banked) to reach a cell density above 2×10^6 cells/mL before transfection to avoid a decrease of transfection efficiency.

Note

Individual culturing and passaging techniques coupled with cellular heterogeneity inherent within the CHO DG44 (cGMP banked) cell population may result in experimental variability.

FreeStyle™ MAX Reagent and OptiPRO™ SFM

FreeStyle™ MAX Reagent

FreeStyle™ MAX Reagent is a proprietary, animal origin-free formulation for the highly efficient transfection of plasmid DNA into eukaryotic cells. FreeStyle™ MAX Reagent is specifically formulated to achieve the highest transfection levels and lowest cytotoxicity in CHO DG44 Cells (cGMP banked) and other suspension cell lines, including FreeStyle™ CHO-S™ and FreeStyle™ 293-F cells.

FreeStyle™ MAX Reagent is also available separately; see page 70 for ordering information.

Store FreeStyle™ MAX Reagent at 2°C to 8°C. Do not freeze.

OptiPRO™ SFM

OptiPRO™ SFM is included with the Freedom™ DG44 Kit to facilitate optimal formation of DNA-lipid complexes. OptiPRO™ SFM is a serum-free medium that is devoid of components of animal or human origin. OptiPRO™ SFM has an ultra-low protein concentration of 7.5 µg/mL.

OptiPRO™ SFM is available separately; see page 70 for ordering information.

Store OptiPRO™ SFM at 2°C to 8°C.

CD OptiCHO™ Medium

Introduction

CD OptiCHO™ Medium is a chemically defined, serum-free medium for selection and high-density suspension culture of stably-transfected CHO DG44 Cells (cGMP banked) expressing DHFR and the neomycin resistance gene. If you are expressing a two-subunit protein, you will perform one round of selection on your transfected cells using CD OptiCHO™ medium containing 500 µg/mL of Geneticin® reagent, as detailed on page 31.

Note: Alternatively, you may perform two rounds of selection to minimize stress, one with CD OptiCHO™ medium and one with CD OptiCHO™ and 500 µg/mL of Geneticin® reagent.

IMPORTANT!

Do not use CD OptiCHO™ Medium or CD OptiCHO™ Medium + Geneticin® reagent to propagate untransfected or parental CHO DG44 Cells (cGMP banked). CHO DG44 Cells (cGMP banked) are DHFR-deficient and require supplementary hypoxanthine and thymidine (HT).

- Only cells that have an active DHFR enzyme, or have been transfected with pOptiVEC™-TOPO® can be propagated in CD OptiCHO™ Medium.
- Only cells that have an active DHFR enzyme, or have been transfected with pOptiVEC™-TOPO® **and** pcDNA™3.3-TOPO® constructs can be propagated in CD OptiCHO™ Medium + Geneticin® reagent.
- We recommend that you use CD DG44 Medium with CHO DG44 Cells (cGMP banked) before they are transfected with pOptiVEC™-TOPO®, which allows them to express the active DHFR enzyme.

Features of the medium

CD OptiCHO™ Medium has the following features:

- Chemically defined, containing no proteins or peptide components of animal, plant, or synthetic origin, and no undefined hydrolysates or lysates
- Formulated without L-glutamine to avoid problems associated with L-glutamine degradation, including ammonia accumulation
- Formulated without phenol red to minimize potential for estrogen-like effects of phenol red

Preparing Complete CD OptiCHO™ Medium

- Supplement CD OptiCHO™ Medium with L-glutamine. Aseptically add L-glutamine to a final concentration of 8 mM to the medium before use.
- For selection with Geneticin® reagent, aseptically add Geneticin® Selective Antibiotic to CD OptiCHO™ Medium at a concentration of 500 µg/mL.
- **Store complete media at 2°C to 8°C protected from light.**

Note

Development work with this kit used 500 µg/mL of Geneticin® reagent; however, because different transfected cells may exhibit different Geneticin® reagent sensitivity, we recommend that you conduct a kill-curve study to establish the ideal concentration of Geneticin® reagent for using with your cells. Note that the cells divide once or twice in the presence of lethal doses of Geneticin® reagent, so the effects of the drug take several days to become apparent.

Methods

Creating expression plasmids for the Freedom™ DG44 Kit

Introduction

The Freedom™ DG44 Kit contains two vectors, pOptiVEC™-TOPO® and pcDNA™3.3-TOPO®. See pages 66–69 for maps and features of each vector.

Using the instructions in this manual, you will:

- For two subunits proteins, clone two separate PCR products corresponding to each of the two subunits (**SU1** and **SU2**, SU: subunit) of your protein of choice separately into pOptiVEC™-TOPO® TA and pcDNA™3.3-TOPO® TA vectors to create **four expression plasmids.** Since individual protein expression may depend on the combination of vectors containing the different subunits of your protein of interest, you will optimize these conditions using different combinations of clones.
- For single subunit proteins, create **an expression plasmid** using the pOptiVEC™-TOPO® TA.

For more information on creating expression plasmids, refer to the instructions in the pOptiVEC™-TOPO® TA and pcDNA™3.3-TOPO® TA Cloning Kit manuals (part nos. 25-0977 and 25-1010, respectively), which are available at **www.lifetechnologies.com/manuals**.

Types of expression plasmids

You will create two expression plasmids in each vector for a total of **four expression plasmids** for two subunit proteins, or create one expression plasmid for a single subunit protein:

Vector	DNA	Selection
pcDNA™3.3-TOPO® TA	mammalian secretion signal and SU1	Geneticin® reagent
pcDNA™3.3-TOPO® TA	mammalian secretion signal and SU2	Geneticin® reagent
pOptiVEC™-TOPO® TA	mammalian secretion signal and SU1	DHFR/HT-
pOptiVEC™-TOPO® TA	mammalian secretion signal and SU2	DHFR/HT-

Recommendation

The combination of vectors for transfection into mammalian cells and the selection process for the Freedom™ DG44 Kit are described on the following pages. For an overview of the experimental steps required to express your protein of interest and the various common experimental pathways you may take while using the Freedom™ DG44 Kit, see **Experimental flowcharts** on pages 8–9.

Thawing and subculturing CHO DG44 Cells (cGMP banked)

Introduction

Follow the protocol below to thaw CHO DG44 Cells (cGMP banked). The cells are supplied in a vial that contains 1 mL of cells at 1×10^7 viable cells/mL in 90% complete CD DG44 medium and 10% DMSO. Thaw the cells directly into CD DG44 Medium supplemented with 8 mM L-glutamine and 18 mL of Pluronic® F-68 per liter.

IMPORTANT!

Do not thaw and grow CHO DG44 Cells (cGMP banked) in CD OptiCHO™ Medium. Parental or untransfected CHO DG44 Cells (cGMP banked) are DHFR-negative and require supplementary hypoxanthine and thymidine, which are present in CD DG44 Medium.

Preparing Complete CD DG44 Medium

- All solutions and equipment that come in contact with the cells must be sterile. Always use proper aseptic technique and work in a laminar flow hood.
- Supplement CD DG44 Medium to a final concentration of 8 mM L-glutamine and 18 mL of 10% Pluronic® F-68 per liter before use (see page 11).
- Addition of antibiotics is not recommended.
- CD DG44 Medium is light sensitive. For optimal results, store medium at 2°C to 8°C, protected from light.

Materials needed

- Frozen CHO DG44 Cells (cGMP banked) (supplied with the kit; store frozen cells in liquid nitrogen until ready to use)
- Complete CD DG44 Medium (prepared as above; pre-warmed to 37°C)
- 125-mL polycarbonate, disposable, sterile Erlenmeyer flasks with vented caps (available from VWR, West Chester PA, Cat. no. 30180-036)
- Orbital shaker set at 130–135 rpm in a 37°C incubator with a humidified atmosphere of 8% CO_2 in air

  Note: This shaking speed (as well as all the values used in other places in this manual) is specific to an Infors Multitron 2 shaker incubator with an orbital throw of 25 mm. When using a shaker with a different orbital throw, we recommend that you modify the shaking speed to match the relative centrifugal force ($RCF = 1.118 \times 10^{-5} \times RADIUS \times SPEED^2$).

Continued on next page

Thawing and subculturing CHO DG44 Cells (cGMP banked), continued

Thawing procedure

1. Remove the cryovial of cells from the liquid nitrogen and thaw quickly (<1 minute) in a 37°C water bath.
2. Decontaminate the outside of the vial with 70% ethanol. Gently break up any clumps with a sterile pipette tip and aseptically transfer the entire contents of the cryovial into a disposable, sterile polycarbonate 125-mL Erlenmeyer shaker flask containing 29 mL of pre-warmed complete CD DG44 Medium.

   Note: Removal of the DMSO from the medium is **not** necessary.
3. Incubate the cells in a 37°C incubator containing a humidified atmosphere of 8% CO_2 in air on an orbital shaker platform rotating at 130–135 rpm.
4. After 24–48 hours in culture, determine the cell density and viability using the protocol described below.
5. After the culture reaches >1.2 × 10^6 viable cells/mL, expand the culture using the subculturing protocol (see next page).

Determining cell density and viability

Follow the procedure below to determine the viable and total cell counts.

1. Transfer a small aliquot of the cell suspension into a microcentrifuge tube.
2. Determine cell viability using your method of choice (such as trypan blue dye exclusion or the Countess® automated cell counter).
3. Determine cell density using your method of choice (such as the Countess® automated cell counter or a Coulter Counter®, or manually using a hemacytometer and an inverted microscope).

Countess® automated cell counter

The Countess® automated cell counter is a benchtop counter designed to measure cell count and viability (live, dead, and total cells) accurately and precisely in less than a minute per sample, using the standard trypan blue technique (see page 71 for ordering information).

A single sample measurement using the Countess® automated cell counter provides the following data within a minute:

- Live and dead cell concentration/mL
- Total cell concentration/mL
- Viability (% live cells to total cells)
- Mean diameter
- Cell images
- Graphical data representation

Continued on next page

Thawing and subculturing CHO DG44 Cells (cGMP banked), continued

Subculturing cells

Passage the cells every 2–3 days into fresh medium. When passaging CHO DG44 Cells (cGMP banked), use disposable, sterile polycarbonate 125-mL Erlenmeyer shaker flasks with vented caps containing 30 mL of pre-warmed complete CD DG44 Medium (for instructions on preparing complete CD DG44 Medium see page 15).

1. Determine the viable and total cell counts.
2. Using the cell density determined in Step 1, calculate the volume of cell culture suspension and fresh medium needed to seed each new shaker flask by dilution. Seed the culture at a density of 3.0×10^5 viable cells/mL, if a subculture step is scheduled for 2 days. Seed the culture at a density of 2.0×10^5 viable cells/mL, if a subculture step is scheduled for 3 days.
3. Transfer the calculated volume of pre-warmed complete CD DG44 Medium into a sterile 125-mL Erlenmeyer shaker flask.
4. Transfer the calculated volume of cell suspension into the pre-warmed complete CD DG44 Medium to give a final cell density of 2×10^5 or 3×10^5 viable cells/mL, depending in the subculture schedule.
5. Incubate the flasks in a 37°C incubator containing a humidified atmosphere of 8% CO_2 in air on an orbital shaker platform rotating at 130–135 rpm.
6. Repeat Steps 1–4 as necessary to maintain or expand cells.

   Note: Do not allow CHO DG44 Cells (cGMP banked) to reach a cell density above 2×10^6 cells/mL before transfection to avoid a decrease of transfection efficiency.

IMPORTANT!

After 25 generations, you should thaw a new vial of cells. To maintain sufficient stocks of low-passage cells (i.e., under 25 generations), be sure to freeze aliquots of CHO DG44 Cells (cGMP banked) in liquid nitrogen. See the next section for instructions on cryopreserving cells.

Freezing CHO DG44 Cells

Introduction

You may freeze CHO DG44 Cells (cGMP banked) directly in CD DG44 Medium with 10% DMSO. We recommend that you freeze the cells at a density of $\geq 1 \times 10^7$ viable cells/mL. Guidelines for preparing freezing medium and to freeze cells are provided in this section.

Materials needed

- Complete CD DG44 Medium
- Tissue culture grade DMSO
- Reagents and equipment to determine viable and total cell counts
- Sterile, labeled cryovials
- Sterile, 15-mL or 50-mL conical tubes
- Automated or manual controlled-rate freezing apparatus

Preparing freezing medium

Prepare freezing medium immediately before use.

1. In a sterile, conical centrifuge tube, mix together the following reagents for every 1 mL of freezing medium needed:

Complete CD DG44 Medium	0.9 mL
DMSO	0.1 mL

   Note: We recommend preparing extra freezing medium to compensate for losses during filtration and pipetting.

2. Filter-sterilize the freezing medium through a 0.22-µm filter and place the tube on ice or store at 2–8°C until use. Discard any remaining freezing medium after use.

Freezing cells

1. Grow the desired quantity of CHO DG44 Cells in shaker flasks, harvesting when the cell density reaches 1×10^6 viable cells/mL with >90% viability. Transfer the cells to a sterile, conical centrifuge tube.
2. Determine the viable and total cell counts (see page 16) and calculate the volume of freezing medium required to yield a final cell density of 1×10^7 viable cells/mL.
3. Centrifuge the cells at $300 \times g$ for 5 minutes at room temperature and carefully aspirate the medium.
4. Resuspend the cells in the pre-determined volume of chilled freezing medium (90% complete CD DG44 medium and 10% DMSO; see above).
5. Place the cryovials in a microcentrifuge rack and aliquot 1 mL of the cell suspension into each cryovial.
6. Freeze the cells in an automated or manual controlled-rate freezing apparatus following standard procedures. For ideal cryopreservation, the freezing rate should be a decrease of 1°C per minute.
7. 24 hours after freezing the cells, transfer the frozen vials to liquid nitrogen for long-term storage.

   Note: You may check the viability and recovery of frozen cells 24 hours after storing the cryovials in liquid nitrogen by following the thawing procedure on page 15.

Establishing sensitivity to Geneticin® Selective Antibiotic (G-418)

Geneticin® Selective Antibiotic (G-418)

The pcDNA™3.3-TOPO® TA vector contains the neomycin resistance gene, which confers resistance to the antibiotic Geneticin® Selective Antibiotic (also known as G-418 sulfate). Geneticin® reagent is available separately (see page 70 for ordering information)

CAUTION!

Geneticin® Selective Antibiotic is harmful. It may cause sensitization by skin contact, and it is irritating to eyes and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Avoid contact with skin and eyes. Wear suitable protective clothing and gloves when handling Geneticin® reagent and Geneticin® reagent-containing solutions.

Preparing and storing Geneticin® Selective Antibiotic

Follow the instructions provided with Geneticin® Selective Antibiotic to prepare your working stock solution. Geneticin® reagent in powder form should be stored at room temperature and at 2°C to 8°C as a solution. The stability of Geneticin® Selective Antibiotic is guaranteed for two years, if stored properly.

Determining Geneticin® Selective Antibiotic sensitivity (Kill-curve study)

The amount of Geneticin® Selective Antibiotic required to be present in culture media to select for resistant cells varies with a number of factors, including cell type. Although the development work with this kit used 500 µg/mL of Geneticin® reagent, we recommend that you re-evaluate the optimal concentration whenever experimental conditions are altered (including use of Geneticin® Selective Antibiotic from a different lot). Note that Geneticin® reagent in powder form has only 75% of the potency of Geneticin® Selective Antibiotic available in liquid form.

The protocol provided below is based on a standard suspension cell culture in a shaker flask. It can be scaled down appropriately to be performed in 6-well, 12-well, or 24-well plate.

1. Seed healthy CHO DG44 cells at 3×10^5 viable cells/mL into 125-mL shaker flasks with fresh growth medium (final volume 30mL) containing varying concentrations of Geneticin® Selective Antibiotic (0, 50, 100, 250, 500, 750, and 1,000 µg/mL of Geneticin® reagent) and culture the cells under standard condition.
2. Sample the cells at regular intervals (for example on days 3, 5, 7, 10, 12, and 14 of culture) to determine viability and viable cell density using your method of choice.
3. Note the percentage of surviving cells at regular intervals to determine the appropriate concentration of Geneticin® reagent that kills the cells (viability <30%) within 10–14 days after addition of Geneticin® Selective Antibiotic.

Methods for two-subunit protein expression

One-page flowcharts

Introduction

The following pages contain flowcharts to aid you in your expression experiments. Each flowchart consists of an outline of the necessary steps and space for notes that you can then transfer to your laboratory notebook.

Using the one-page flowcharts

The page numbers by the experimental steps in the flowcharts are hyperlinked to detailed protocols. Hold down the CTRL key as you click on the page number given in the flowchart to access the appropriate detailed protocol. You may also print the individual flowcharts for use in the laboratory.

Note

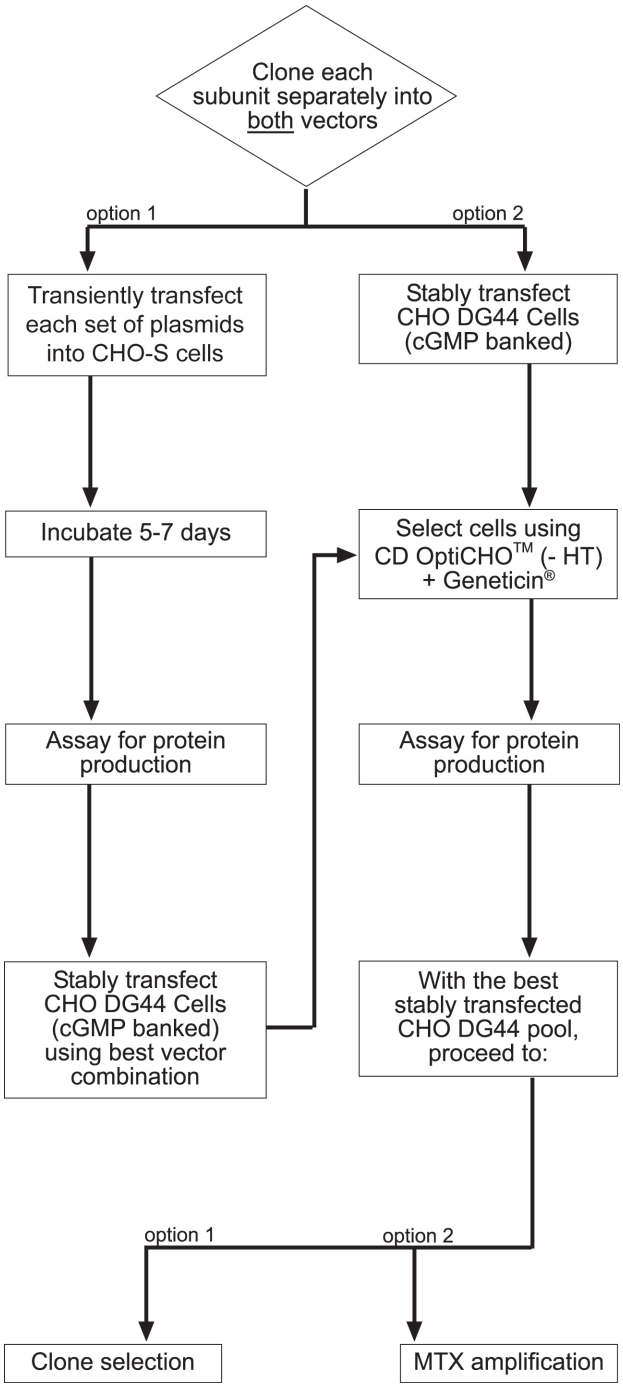
The one-page protocols are experimental guidelines only; they do **not** provide detailed protocols to successfully perform the steps necessary for expressing your protein of interest. Note that the times shown for various experimental steps are approximations; the actual times depend on your protein of interest and the specific workflows you choose.

We recommend that you familiarize yourself with the detailed protocols before starting your experiments.

Optimizing vectors for two-subunit protein expression

Introduction

Prior to making stable transfectants in CHO DG44 Cells (cGMP banked), you may perform transient transfections of CHO-S cells or several stable transfections of CHO DG44 Cells (cGMP banked) with various combinations of pOptiVEC™ and pcDNA™3.3 plasmid constructs to determine which vector combination gives optimal protein yield.

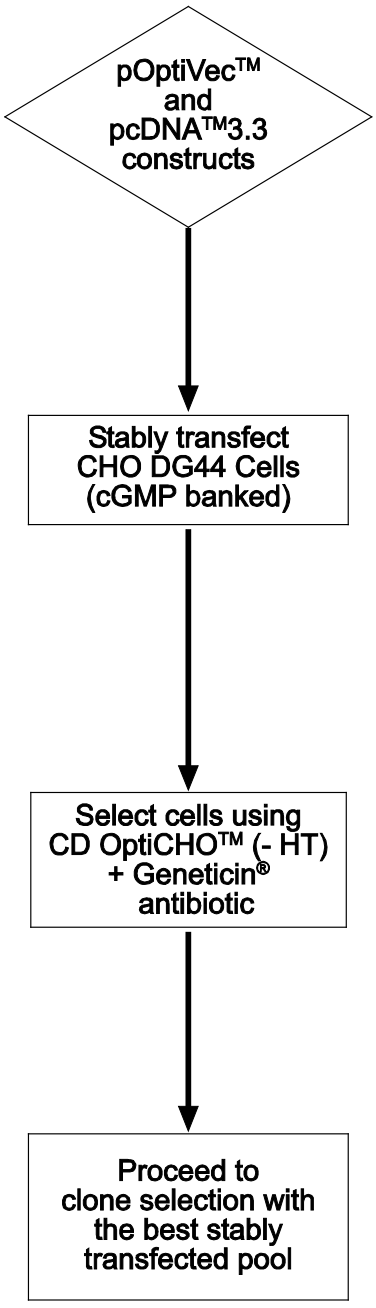

Clone each subunit separately into both vectors
option 1
option 2
Transiently transfect each set of plasmids into CHO-S cells
Stably transfect CHO DG44 Cells (cGMP banked)
Incubate 5-7 days
Select cells using CD OptiCHO™ (- HT) + Geneticin®
Assay for protein production
Assay for protein production
Stably transfect CHO DG44 Cells (cGMP banked) using best vector combination
With the best stably transfected CHO DG44 pool, proceed to:
option 1
option 2
Clone selection
MTX amplification

Page	Time Line	Notes
page 14	Molecular cloning: ~ 1 week	
Transient: page 25 Stable: page 26	Transfection: 1 day	
page 28	Selecting stable transfectants: 14–21 days	
page 33	Protein assays: usually 7–10 days	
	Transfection: 1 day	
Clone selection: page 37		
MTX amplification: page 35		

Expressing two-subunit proteins without gene amplification

Introduction

The flowchart below depicts the major steps to express your two-subunit protein in suspension CHO DG44 Cells (cGMP banked) with the combination of pOptiVEC™ and pcDNA™3.3 constructs that gave you the highest yield.

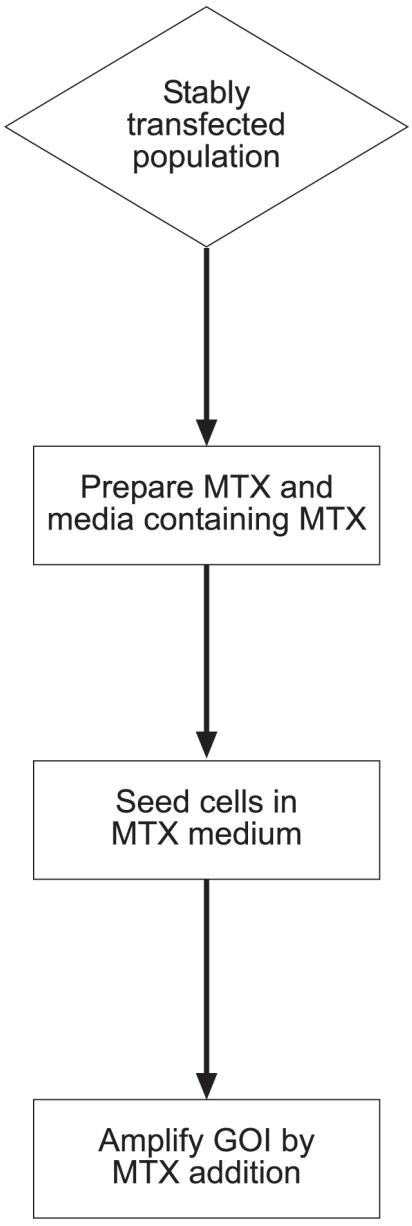

pOptiVec™ and pcDNA™3.3 constructs
Stably transfect CHO DG44 Cells (cGMP banked)
Select cells using CD OptiCHO™ (- HT) + Geneticin® antibiotic
Proceed to clone selection with the best stably transfected pool

	Page	Time Line	Notes
Stably transfect CHO DG44 Cells (cGMP banked)	page 26	Transfection: 1 day	
Select cells using CD OptiCHO™ (- HT) + Geneticin® antibiotic	page 28	Start 2–3 days post-transfection; usually lasts 12–14 days	
Proceed to clone selection with the best stably transfected pool	page 37	Clone selection: 12–14 days	

Genomic amplification by MTX addition

Introduction

The flowchart below depicts the major steps to amplify the copy number of your gene of interest (GOI) using MTX (as methotrexate hydrate) for increased production of the protein of interest.

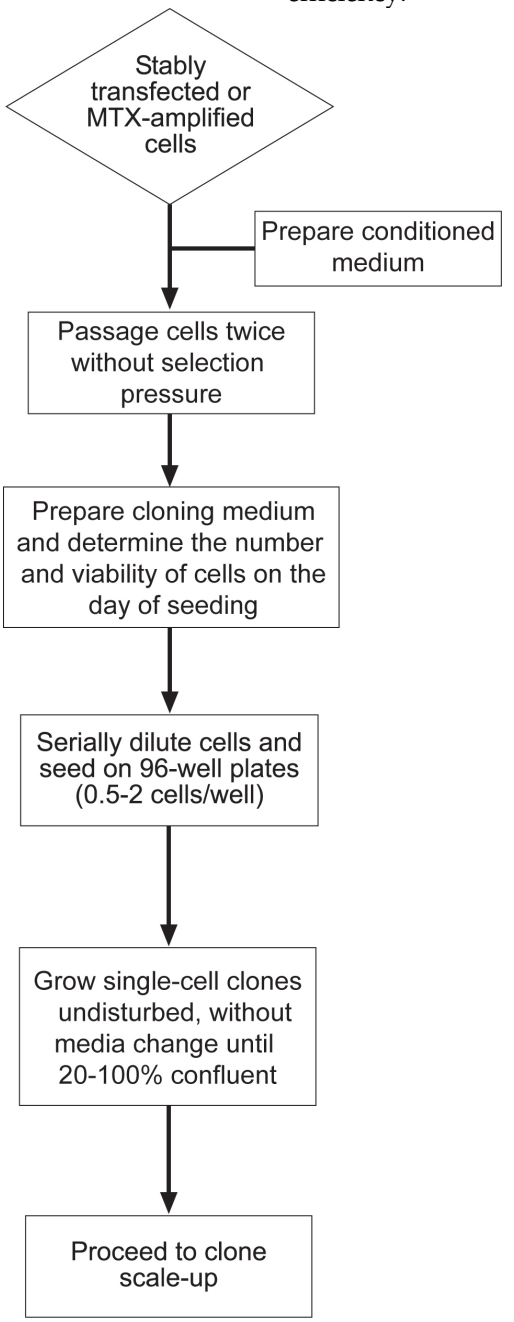

Stably transfected population
Prepare MTX and media containing MTX
Seed cells in MTX medium
Amplify GOI by MTX addition

	Page	Time Line	Notes
Prepare MTX and media containing MTX	page 35	Media prep: 1–3 hours	
Seed cells in MTX medium	page 36	Seeding cells: 1 hour	
Amplify GOI by MTX addition	page 36	2–4 weeks (variable depending on protein of interest)	

Performing clonal selection by limiting dilution (two-subunit protein)

Introduction

The flowchart below depicts the major steps to obtain a clonal cell line (i.e., derived from a single cell) for the production of a two-subunit protein by diluting the pool of stably transfected cells or the MTX-amplified cells to 0.5–2 cells per well in a 96-well plate containing cloning medium. In most cases, one of the cells forms a distinct colony that can later be scaled up. You may also statistically calculate the desired number of cells per well to help ensure monoclonality. However, using a lower seeding density may result in decreased cloning efficiency.


Stably transfected or MTX-amplified cells
Prepare conditioned medium
Passage cells twice without selection pressure
Prepare cloning medium and determine the number and viability of cells on the day of seeding
Serially dilute cells and seed on 96-well plates (0.5-2 cells/well)
Grow single-cell clones undisturbed, without media change until 20-100% confluent
Proceed to clone scale-up

Page	Time Line	Notes
page 37	3–5 days	
	5–7 days	
pages 38–39	2 hours	
page 39	4 hours	
page 40	10–14 days	
page 41		

Transiently transfecting FreeStyle™ CHO-S™ cells

Recommendation

We recommend FreeStyle™ CHO-S™ cells and transfection with FreeStyle™ MAX Reagent for **transient transfection to determine which vector combination gives optimal protein yield**. Both FreeStyle™ CHO-S™ cells and FreeStyle™ CHO Expression Medium are available separately, see page 70.

Plasmid preparation

The pOptiVEC™ and pcDNA™3.3 plasmid constructs must be clean, sterile, and free from contamination with phenol and sodium chloride for transfection into cells. Contaminants may kill the cells, and salt interferes with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using an endotoxin-free or a low-endotoxin kit such as the PureLink® HiPure Plasmid Midiprep DNA Kit (see page 71 for ordering information).

Note: Plasmids may be linearized or circular for transient transfection. For stable transfection of CHO DG44 Cells (cGMP banked), we recommend linearizing the plasmid, see page 26.

General guidelines for transient transfection

To transiently transfect FreeStyle™ CHO-S™ cells, use equal amounts of each pOptiVEC™ and pcDNA™3.3 plasmid DNA constructs containing each subunit of your protein of interest and follow the recommended protocol included with your CHO cells and transfection reagent. After transfection, culture cells for 5–7 days (no medium change is required) and assay for protein expression using your method of choice (see below).

Assessing protein production

To check for expression of your protein after transient transfection, you may take an aliquot of growth media and perform SDS-PAGE, protein-specific ELISA, or the bioactivity assay of choice to determine that your cells are producing your protein of interest.

Transfecting CHO DG44 Cells with FreeStyle™ MAX Reagent for two-subunit protein expression

Introduction

After determining which combination of pOptiVEC™ and pcDNA™3.3 plasmid constructs gives optimal protein yield using your detection method of choice, you will use FreeStyle™ MAX Reagent to stably transfect suspension CHO DG44 Cells (cGMP banked) with the best combination of vectors. If the data from transient transfection do not clearly demonstrate one option over the other, or if your resources allow, you may transfect the cells with both vector combinations.

Plasmid preparation

The pOptiVEC™ and pcDNA™3.3 plasmid constructs must be clean, sterile, and free from contamination with phenol and sodium chloride for transfection into CHO DG44 Cells (cGMP banked). Contaminants may kill the cells, and salt interferes with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using an endotoxin-free or a low-endotoxin kit such as the PureLink® HiPure Plasmid Midiprep DNA Kit (see page 71 for ordering information).

Linearizing the plasmids

Prior to using the Freedom™ DG44 Kit to transfect CHO DG44 Cells (cGMP banked) with your pOptiVEC™ and pcDNA™3.3 constructs, you may linearize the plasmids. Linearizing your vectors may not improve transfection efficiency, but it increases the chance that the vectors will integrate into the host cell genome without disrupting the gene of interest or other elements required for expression in mammalian cells. Follow the guidelines below to linearize your plasmids.

- We suggest using *Pvu* I, which cuts once in the ampicillin resistance gene on each plasmid. Other unique restriction sites are possible. Complete restriction maps of pOptiVEC™-TOPO® TA and pcDNA™3.3-TOPO® TA are available at **www.lifetechnologies.com**. *Be sure that your inserts do not contain the restriction enzyme site you use to linearize the vector.*

  Note: If an appropriate linearization site is not present, you may transfect the circular plasmid. Transfection efficiency will not be affected.
- After digestion, precipitate the DNA, resuspend pellet in sterile water, and re-quantify using your method of choice.

Recommendation

Calculate the number of CHO DG44 Cells (cGMP banked) that you will need for your transfection experiment and expand cells accordingly. Make sure that the cells are healthy and greater than 95% viable before proceeding to transfection.

Materials needed

- Suspension CHO DG44 Cells (cGMP banked) cultured in complete CD DG44 Medium at 5×10^5 viable cells/mL
- Purified, linearized pOptiVEC™ and pcDNA™3.3 plasmid DNA containing the subunits of your gene of interest, prepared as explained on page 26
- FreeStyle™ MAX Reagent (supplied with the kit; store at 2°C to 8°C until use)
- OptiPRO™ SFM (supplied with the kit; pre-warmed to room temperature)
- Disposable, sterile, 125-mL polycarbonate Erlenmeyer flasks
- Orbital shaker in 37°C incubator with a humidified atmosphere of 8% CO_2
- Reagents and equipment to determine viable and total cell counts (e.g., Trypan Blue, hemacytometer, or an automated cell counter such as the Countess® Automated Cell Counter or similar)

Continued on next page

Transfecting CHO DG44 Cells with FreeStyle™ MAX Reagent for two-subunit protein expression, continued

Optimal transfection conditions

To transfect suspension CHO DG44 Cells (cGMP banked) in a 30 mL volume, we recommend using the following optimized conditions:

- **Final transfection volume:** 30 mL
- **Number of cells to transfect:** total of 1.5×10^7 viable cells (cell density at time of transfection should be 5×10^5 viable cells/mL)
- **Amount of each plasmid DNA:** 9 µg each (total 18 µg)
- **FreeStyle™ MAX Reagent:** 15 µL

Note: Further optimization of culture volume or transfection conditions is not necessary for stable cell line production.

Transfection procedure for expression of two-subunit protein

Follow the procedure below to transfect CHO DG44 Cells (cGMP banked) in a **30-mL volume**. We recommend including negative controls (no FreeStyle™ MAX Reagent, no DNA) in your experiment to help you evaluate your results.

1. At 48 hours before transfection, pass CHO DG44 Cells (cGMP banked) at 3×10^5 viable cells/mL in complete CD DG44 Medium. Place the flask(s) on an orbital shaker platform rotating at 130–135 rpm at 37°C, 8% CO_2.
2. At 24 hours before transfection, pass CHO DG44 Cells (cGMP banked) at 3×10^5 viable cells/mL in complete CD DG44 Medium. Place the flask(s) on an orbital shaker platform rotating at 130–135 rpm at 37°C, 8% CO_2.
3. On the day of transfection, perform a viable cell count. To ensure optimal transfection results, viability of cells must be over 95%.
4. For each transfection or control, transfer 1.5×10^7 viable CHO DG44 Cells (cGMP banked) into a new 125-mL flask. Add pre-warmed, complete CD DG44 Medium to a final volume of 30 mL. Place the flask in shaker until ready to transfect.

   Note: Do not centrifuge cells prior to transfection, because centrifugation decreases transfection efficiency.
5. Gently invert the tube of FreeStyle™ MAX Reagent several times to mix. **Do not vortex.**
6. Add 18 µg of plasmid DNA (9 µg of each construct) to 600 µL of OptiPRO™ SFM, and mix gently. Add 15 µL of FreeStyle™ MAX Reagent into 600 µL of OptiPRO™ SFM, and mix gently.
7. Immediately add diluted FreeStyle™ MAX Reagent solution to the diluted DNA solution and mix gently. Incubate the DNA-FreeStyle™ MAX mix for 10 minutes at room temperature to allow complexes to form. Do **not** incubate for longer than 20 minutes.
8. Drop-wise add 1.2 mL of DNA-FreeStyle™ MAX Reagent complex into the 125-mL flask containing cells while slowly swirling the flask.
9. Incubate the transfected cell cultures at 37°C, 8% CO_2 on an orbital shaker platform rotating at 130–135 rpm.
10. 48 hours after transfection, pass the cells into HT-deficient, complete CD OptiCHO™ Medium (see page 13). Proceed to **Selecting Stable Transfectants for Two-subunit Protein Expression**, page 31.

Transfecting CHO DG44 Cells with Neon® for two-subunit protein expression (optional)

Recommendation

Calculate the number of CHO DG44 Cells (cGMP banked) that you will need for your transfection experiment and expand cells accordingly. Make sure that the cells are healthy and greater than 95% viable before proceeding to transfection.

Materials needed

- Suspension CHO DG44 Cells (cGMP banked) cultured in complete CD DG44 Medium
- Purified, linearized pOptiVEC™ and pcDNA™3.3 plasmid DNA containing the subunits of your gene of interest, prepared as detailed on page 26
- Neon® Transfection system (see page 71 for ordering information)

  Note: Resuspension Buffers R and E2 used in this protocol are Animal-Origin Free (AOF).
- Disposable, sterile T-75 tissue culture flasks
- A static culture incubator at 37°C with a humidified atmosphere of 8% CO_2
- Reagents and equipment to determine viable and total cell counts (e.g., Trypan Blue, hemacytometer, or an automated cell counter such as the Countess® Automated Cell Counter)

Optimal transfection conditions

To transfect suspension CHO DG44 Cells (cGMP banked) in a 15–20 mL volume, we recommend using the following optimized conditions:

- **Number of cells to transfect:** total of 1.0×10^7 viable cells (in 100 µL Neon® tip)
- **Amount of each plasmid DNA:** 10 µg each (total 20 µg)
- **Neon® Electroporation program:** #5, #9 or #24

  Note: We observed equivalent transfection efficiency (≥ 85%) with the three Neon® programs above at 24 hours and 48 hours post-transfection. We recommend using these programs as a starting point to test the stable transfection of your gene of interest.

General guidelines of transfection

- Prepare high-quality plasmid DNA at a concentration of 1–5 µg/µL in deionized water or TE buffer.
- Use an appropriate GFP plasmid transfected in parallel to determine transfection efficiency if possible.
- Discard the Neon® Tips after two uses and Neon® Tubes after 10 uses as a biological hazard. Change the tube and buffer when switching to a different plasmid DNA or cell type.
- The plasmid DNA amount should not exceed 10% of total volume used for transfection.
- Prepare extra volume of cell suspension to ensure that the desired volume is available at the time of transfection.

Continued on next page

Transfecting CHO DG44 Cells with Neon® for two-subunit protein expression, continued

Preparing cells

1. 24 hours before transfection, passage CHO DG44 cells at 3×10^5 viable cells/mL in complete CD DG44 medium. Place the flask(s) on an orbital shaker platform rotating at 130–135 rpm at 37°C, 80% relative humidity, and 8% CO_2
2. On the day of transfection, harvest appropriate amount of cells (total of 1.0×10^7 viable cells per transfection) and wash the cells in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}.
3. Resuspend the cell pellet in appropriate volume (100 µL per transfection) of Resuspension Buffer R (included with Neon® Kits) at a final density of 1.0×10^8 cells/mL.
4. Prepare T-75 flask(s) by filling the flask(s) with 15–20 mL of complete CD DG44 medium without antibiotics and pre-incubate flask(s) at 37°C in an 80% relative humidity, 8% CO_2 incubator for at least 15 minutes.

Transfection procedure for expression of two-subunit protein

1. Fill the Neon® Tube with 3 mL of Electrolytic Buffer E2 (for a 100 µL Neon® tip).
2. Insert the Neon® Tube into the Neon® Pipette Station until you hear a click.
3. Transfer the appropriate amount of plasmid DNA (20 µg DNA per transfection) into a sterile, 1.5 mL microcentrifuge tube.
4. Add appropriate amount of cells (100 µL per transfection) to the tube containing plasmid DNA and gently mix.
5. Press the push-button on the Neon® Pipette to the second stop to open the clamp and insert the top-head of the Neon® Pipette into the Neon® Tip until the clamp fully picks up the mounting stem of the piston. Gently release the push-button, continuing to apply a downward pressure on the pipette, ensuring that the tip is sealed onto the pipette without any gaps.
6. Press the push-button on the Neon® Pipette to the first stop and immerse the Neon® Tip into the cell-DNA mixture. Slowly release the push-button on the pipette to aspirate the cell-DNA mixture into the Neon® Tip.
7. Insert the Neon® Pipette with the sample vertically into the Neon® Tube placed in the Neon® Pipette Station until you hear a click.
8. Ensure that you have selected the appropriate electroporation protocol and press **Start** on the touchscreen.

   The Neon® device automatically checks for the proper insertion of the Neon® Tube and Neon® Pipette before delivering the electric pulse.

   The touchscreen displays "Complete" to indicate that electroporation is complete.

Continued on next page

Transfection procedure for expression of two-subunit protein, continued

9. Remove the Neon® Pipette from the Neon® Pipette Station and immediately transfer the samples from the Neon® Tip by pressing the push-button on the pipette to the first stop into the prepared T-75 flask(s) containing pre-warmed medium without antibiotics. Discard the Neon® Tip into an appropriate biological hazardous waste container.
10. Repeat Steps 5–9 for the remaining samples. Change the Neon® Tips after two uses and Neon® Tubes after ten uses. Use a new Neon® Tip and Neon® Tube for each new plasmid DNA or cell type.
11. Gently rock the T-flask to assure even distribution of the cells. Incubate the flask at 37°C in a humidified CO_2 incubator.
12. If you are not using the Neon® device, turn the power switch on the rear to "OFF".
13. 48 hours after transfection, centrifuge the cells at 300 × *g* for 5 minutes and remove the medium by aspiration. Resuspend cells with 30 mL of pre-warmed complete CD OptiCHO™ Medium containing 500 µg/mL of Geneticin® reagent to give a final cell density of 5×10^5 viable cells/mL in a 125-mL shaker flask. Proceed to **Selecting Stable Transfectants for Two-subunit Protein Expression**, next page.

Selecting stable transfectants for two-subunit protein expression

Introduction

To obtain cell lines that produce high levels of your protein, first select for a pool of stably-transfected cells, in which the linearized pOptiVEC™ and pcDNA™3.3 constructs have integrated into the host cell genome. Perform the selection using complete CD OptiCHO™ Medium containing 500 µg/mL of Geneticin® Selective Antibiotic. Note that only cells that have been transfected with pOptiVEC™ **and** pcDNA™3.3 constructs can be propagated in CD OptiCHO™ Medium + Geneticin® reagent, because untransfected CHO DG44 Cells (cGMP banked) lack APH and DHFR activities (see below).

Geneticin® Selective Antibiotic

Geneticin® Selective Antibiotic (G-418) blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression in mammalian cells of the bacterial gene (APH), derived from Tn*5*, results in detoxification of Geneticin® reagent (Southern & Berg, 1982).

Calculate the concentration based on the amount of active drug. Cells will divide once or twice in the presence of lethal doses of Geneticin® reagent, so the effects of the drug take several days to become apparent. Complete selection can take up to two weeks of growth in selective medium.

Note

Development work with this kit used 500 µg/mL of Geneticin® reagent; however, because different transfected cells may exhibit different Geneticin® Selective Antibiotic sensitivity, we recommend that you conduct a kill-curve study to establish the ideal concentration of Geneticin® reagent for using with your cells. See page 19, **Establishing Sensitivity to Geneticin® Selective Antibiotic (G-418)**, for a kill-curve study protocol.

DHFR

DHFR catalyzes the reduction of 5, 6-dihydrofolate to 5, 6, 7, 8-tetrahydrofolate, which is essential for DNA synthesis. CHO-derived CHO DG44 Cells (cGMP banked) lack DHFR activity, and they must be propagated in medium containing the purine precursors hypoxanthine and thymidine (HT) unless the cells are stably transfected with a vector that expresses DHFR.

Continued on next page

Selecting stable transfectants for two-subunit protein expression, continued

Selecting stable transfectants for two-subunit protein expression

48 hours after transfection, passage transfected CHO DG44 Cells (cGMP banked) in complete CD OptiCHO™ Medium containing 500 µg/mL of Geneticin® reagent to select for stably transfected clones. To passage cells:

1. Determine viable and total cell counts (see page 16).
2. Dilute the cells in pre-warmed complete CD OptiCHO™ Medium containing 500 µg/mL of Geneticin® reagent to give a final cell density of 5×10^5 viable cells/mL.
3. Incubate flasks in a 37°C incubator containing a humidified atmosphere of 8% CO_2 on an orbital shaker platform rotating at 130–135 rpm.
4. Centrifuge cells at 300 × *g* for 5 minutes, remove the medium by aspiration, and add fresh medium to the desired final volume every 3–4 days for 14–21 days until cell viability increases to >90% (see **Note** below). It is not necessary to centrifuge the cells and re-suspend them in complete fresh medium if the dilution factor at the time of passage is >2.
5. When the culture reaches >90% viability, maintain it at 3×10^5 viable cells/mL and scale up the culture as needed.

Note

During the selection round, cell viability may drop dramatically (to <10%) due to the death of untransfected and transiently-transfected cells. To promote optimal growth of stably transfected cells, maintain cultures as described in Steps 4–5.

Assessing productivity

Protein production

To check for production of your protein during stable cell establishment, you may take an aliquot of growth media and perform SDS-PAGE, protein-specific ELISA, or the bioactivity assay of choice to determine that your cells are producing your protein of interest.

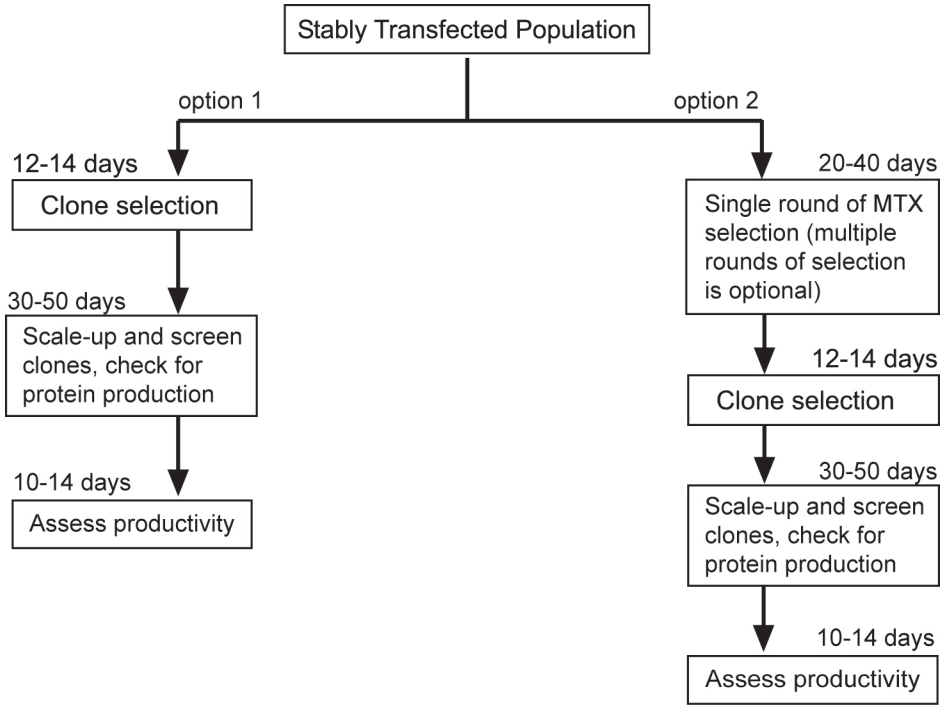
Important

When you have a pool of stably-transfected cells, freeze several aliquots of the pool using the procedure on page 18.

Choosing a workflow

At this stage you will have a population of stably-transfected CHO DG44 Cells expressing your protein of interest at various levels. For most bioproduction applications, several clonally-derived cell lines producing your protein are desirable for screening. However, the productivity of each clone depends upon the integration locus of the plasmid(s), the response to amplification using MTX, and the nature of the protein.

Depending on your protein production needs, the time and effort required to generate clonal, high-producing cell lines also vary. Two common pathways from stable pool to clone scale-up are outlined below. **Note that the times shown for various experimental steps are approximations, and the actual times depend on your protein of interest and the specific workflows you choose.**



Continued on next page

Assessing productivity, continued

Points to consider

When choosing a workflow, consider the amount of protein you wish to produce, your available resources, and the amount of time it will take to obtain your clonal, high-producing cell lines. Because MTX amplification produces a polyclonal population, you must always perform clone selection prior to scale-up.

Additional cloning media, supplements, and other products may be purchased separately (page 70).

Assessing productivity

The following protocol is used to assess the productivity in batch culture. Nutrition feed protocol can be added appropriately if needed.

1. Seed fully recovered cells (viability >90%) at 3×10^5 viable cells/mL with 30 mL fresh medium (CD OptiCHO™ media supplemented with 4 mM L-glutamine) in 125-mL shaker flasks. The culture volume can be scaled up based on the schedule of sampling.

   Note: You can adjust the L-glutamine concentration as needed (for example 0–8 mM).

2. Sample cultures daily or at regular intervals (for example, on Day 0, 3, 5, 7, 10, 12, and 14) until culture viability drops below 50% (whichever comes first) to determine the cell density, viability, and productivity.

   Keep the cell seed-train growing according to the subculture protocol until you successfully complete your productivity study.

Next steps

- To perform 1 round of genomic amplification using MTX selection to obtain a population of cells expressing high levels of your protein, see page 35.
- To perform limiting dilution cloning to obtain single clones expressing high levels of your protein, see page 37.
- To scale up your clones for protein expression, see page 41.

Genomic amplification by MTX addition

Introduction

Methotrexate (MTX) is a folic acid antagonist that is actively transported into cells by the folate transporter. In the cell, it is converted to a high molecular weight polyglutamate metabolite by folylpolyglutamate synthase, which binds to DHFR and inhibits its activity.

If MTX is present in the medium, cells compensate by increasing the DHFR copy number in the genome to overcome inhibition by MTX. Since the gene of interest is integrated into the same genetic locus as DHFR, the gene of interest is amplified as well, leading to increased production of the protein of interest (Kaufman *et al.*, 1985; Tanaka *et al.*, 2002). MTX (as methotrexate hydrate) is available from Sigma (10 mg, Cat. no. A6770).

CAUTION!

MTX is toxic to the skin, eyes, and respiratory system. Wear suitable protective clothing, gloves, and eye and face protection when working with MTX. Refer to the product MSDS for complete precautions.

Preparing 1 mM MTX

To prepare a 1 mM MTX stock solution:

1. Dissolve 10 mg MTX in 22 mL of PBS.
2. Filter-sterilize the solution through a 0.22-µm filter.
3. Store in 250 µL aliquots at –20°C.

Preparing media with MTX

- To make complete CD OptiCHO™ Medium containing MTX, use complete CD OptiCHO™ Medium (prepared with L-glutamine) plus the required concentration of MTX. If you amplifying a two-subunit protein, you must also add 500 µg/mL of Geneticin® reagent to the complete CD OptiCHO™ Medium.
- Using the sterile, 1 mM MTX stock solution (prepared as described above), prepare media containing the appropriate concentration of MTX.

Note

Development work with this kit used 500 µg/mL of Geneticin® reagent; however, because different transfected cells may exhibit different Geneticin® reagent sensitivity, we recommend that you conduct a kill-curve study to establish the ideal concentration of Geneticin® Selective Antibiotic to use with your cells. See page 19, **Establishing Sensitivity to Geneticin® Selective Antibiotic (G-418)**, for a kill-curve study protocol.

Continued on next page

Genomic amplification by MTX addition, continued

One round of MTX amplification

The productivity of each clone depends upon the integration locus of your expression construct, the response to amplification using MTX, and the nature of your protein. Depending on your protein production needs, your available time, and your resources, you may perform one round of MTX amplification at various concentrations (such as 50 nM, 100 nM, 250 nM, 500 nM, and 1 µM). Additional rounds of MTX amplification may be performed using higher concentrations (such as 2 µM and 4 µM) to potentially increase your protein production.

Protocol for MTX amplification

1. For each cell pool, centrifuge cells at 300 × *g* for 5 minutes, then aspirate old medium.
2. Seed cells at a density of 3×10^5 viable cells/mL in 100–300 mL of media containing various concentrations of MTX in 0.5–1 liter shaker flasks.
3. Incubate flasks at 37°C/8% CO_2 with shaking at 130–135 rpm.
4. Passage cells into fresh medium containing MTX at 2×10^5–3×10^5 viable cells/mL in shaker flasks every 3 or 4 days. Spin down cells and re-suspend cells in fresh medium if the dilution factor at passage is <2.
5. Passage cells at 2×10^5–3×10^5 viable cells/mL when the viability starts increasing. The cells amplified with low concentration of MTX recover faster than that with high concentration of MTX.
6. When cell viability is >90%, freeze cells and start protein productivity analysis.

Next steps

- Because MTX amplification produces a polyclonal population of cells, you must always perform clone selection (page 37) prior to clone scale-up.
- *Optional*: You may perform further evaluation of the MTX amplified pools to decide which amplified pool to choose for clone selection and/or protein production.

Clonal selection by limiting dilution

Introduction

Development of a CHO cell line for commercial production of a recombinant protein requires clonality of the final cell population. This is achieved by limiting dilution cloning (LDC). Before performing LDC, expand the stably transfected or MTX-amplified cells in CD OptiCHO™ medium supplemented with 8 mM glutamine and without any selection pressure for at least two passages. On the day of cloning, dilute the cells to seed 0.5–2 cells per well in a 96-well plate. In most cases, one of the cells forms a distinct colony that can be scaled up using the procedure described below. You may also statistically calculate the desired number of cells per well to help ensure clonality of the colonies. However, using a lower seeding density may result in decreased cloning efficiency.

Cloning medium considerations

- Because the growth rate and protein production of each clone vary, you may need to optimize clonal selection conditions by adjusting the number of cells per well, the volume of the cloning medium, and various media supplements.
- CHO DG44 transfected pools achieve good cloning efficiency when CD FortiCHO™ Medium is used as the cloning medium.
- To achieve the highest cloning efficiency in LDC, CHO DG44 transfected pools require the addition of conditioned media and HT Supplement. See page 71 for ordering information.
- Depending on how many clones you wish to screen before scale-up, you may increase the number of limiting dilution plates according to your anticipated cloning efficiency and level of protein production. The number of clones obtained from a 96-well plate varies depending on the experiment.

Preparing conditioned medium

You can collect spent medium from a transfected CHO DG44 pool and use it as conditioned medium. We recommend using DG44 cells transfected with an empty pOptiVEC™ vector, which you can generate by inserting a small PCR fragment to close the TOPO cloning site. The advantage of this method is that you can use the conditioned medium for cloning with any transfected parental DG44 pool. Freeze the transfected cell pool and use as needed to generate conditioned medium.

1. Seed 3×10^5 viable cells/mL in a sufficient culture volume of CD OptiCHO™ medium supplemented with 8 mM glutamine to generate enough conditioned medium needed for cloning experiments.
2. Grow the cells in batch culture for 5 days.
3. Centrifuge the cultures and collect the supernatant (spent medium). Discard the cell pellet.
4. Sterilize the collected spent medium by membrane filtration.
5. Freeze the condition medium in aliquots at –80°C until needed.

Continued on next page

Clonal selection by limiting dilution, continued

Preparing cloning medium

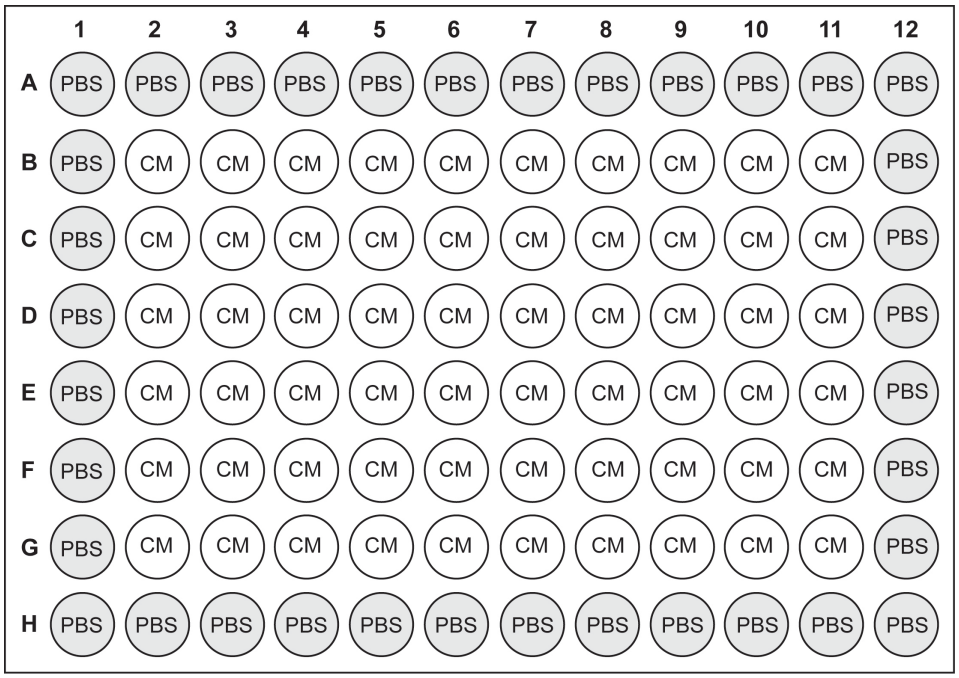
The procedure described below uses CD FortiCHO™ Medium as the cloning medium. One 50-mL conical tube is sufficient to seed approximately 200 wells at 1 cell/well. For greater numbers of wells or plates, adjust the volumes accordingly.

1. Thaw L-glutamine to be used in preparation of completed cloning medium.
2. For each 100 mL of completed cloning medium required, aseptically mix the following:

   86 mL of basal CD FortiCHO™ Medium
   3 mL of freshly thawed 200 mM L-glutamine
   10 mL of conditioned medium
   1 mL of 100X HT supplement
3. For each 40 mL of cells required for plating, add 39.8 mL of completed cloning medium to a 50-mL centrifuge tube and mix by gentle inversion 5–6 times.
4. Pre-warm the medium at 37°C for one hour before using it for plating the cells (page 40).

Plate setup

1. Label a sufficient number of 96 well plates for your procedure.
2. Add 200 µL/well of sterile PBS to all peripheral wells to avoid evaporation during incubation (as shown in the figure below).


1 2 3 4 5 6 7 8 9 10 11 12
A PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS
B PBS CM CM CM CM CM CM CM CM CM CM PBS
C PBS CM CM CM CM CM CM CM CM CM CM PBS
D PBS CM CM CM CM CM CM CM CM CM CM PBS
E PBS CM CM CM CM CM CM CM CM CM CM PBS
F PBS CM CM CM CM CM CM CM CM CM CM PBS
G PBS CM CM CM CM CM CM CM CM CM CM PBS
H PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS

CM = cells diluted in **cl**oning **m**edium

Continued on next page

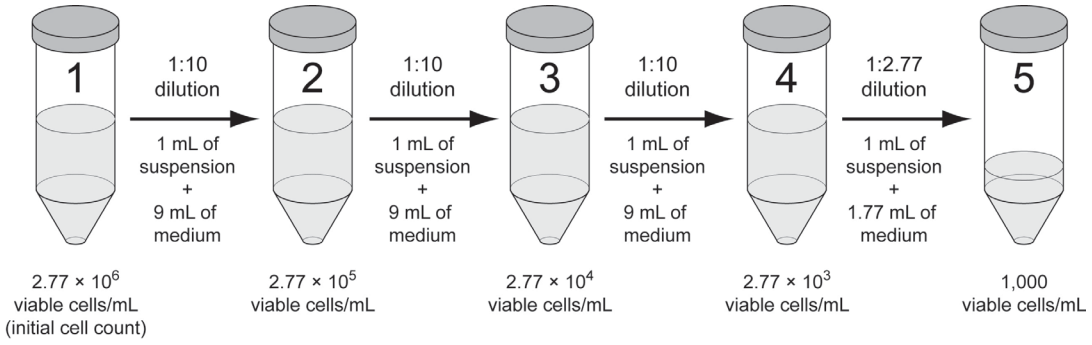
Clonal selection by limiting dilution, continued

Cell counting and dilution

1. Label five 50-mL conical tubes “1” through “5”.
2. Pipette 5 to 10 mL of your transfected pool CHO DG44 cells into the 50-mL tube labeled “1”.
3. Determine the viable cells/mL accurately using your method of choice.
4. Serially dilute the cells to a final concentration of 1,000 viable cells/mL using growth medium (CD OptiCHO™ Medium supplemented with 8 mM L-glutamine) as shown in figure below to yield a seeding density of 1 viable cell/well. If a different cell/well seeding density is desired, adjust accordingly in the final dilution step.

   Note: Mix cells gently after each dilution by inverting the capped tube 5 to 6 times. Avoid foaming.

Example of cells serially diluted in CD OptiCHO™ Medium with an initial count of 2.77×10^6 viable cells/mL


1
1:10 dilution
1 mL of suspension + 9 mL of medium
2
1:10 dilution
1 mL of suspension + 9 mL of medium
3
1:10 dilution
1 mL of suspension + 9 mL of medium
4
1:2.77 dilution
1 mL of suspension + 1.77 mL of medium
5
2.77 × 10^6 viable cells/mL (initial cell count)
2.77 × 10^5 viable cells/mL
2.77 × 10^4 viable cells/mL
2.77 × 10^3 viable cells/mL
1,000 viable cells/mL

Continued on next page

Clonal selection by limiting dilution, continued

Plating cells

1. After warming the cloning medium (step 4, page 38), remove it from the incubator and place it in the laminar flow hood.
2. Pipette 0.2 mL of the cell suspension from "Tube 5" (1,000 cells/mL) into the cloning medium.
3. Mix the cell suspension gently by inverting the tube 5 or 6 times and transfer it into a sterile reagent reservoir or trough.
4. Aseptically dispense 200 µL of the diluted cells into each of the empty 60 wells of each 96-well plate, using a multi-channel pipettor.
5. Incubate the plates **undisturbed** for 10–14 days at 37°C and 5% CO_2 in humidified air in a static (non-shaking) incubator. Stack no more than 5 plates together.
6. After day 10 of incubation, examine the wells visually using a microscope for growth of monoclonal colonies.
7. Perform your primary screen (i.e., protein assay of choice) to determine the cloning efficiency and identify clones of interest.
8. Calculate the percent cloning efficiency, normalized at 1 cell per well, as follows

$$\text{Cloning Efficiency} = \frac{\text{Number of wells showing growth of a single colony}}{\text{Number of wells seeded} \times \text{cells per well seeded}} \times 100\%$$

For example, the cloning efficiency with an experiment of 120 single colonies growing out of total 600 seeded wells (10 plates with 60 seeded wells per plate) at a seeding density of 0.5 cell per well is 40%.

Next steps

After testing these clones for high levels of protein production using your method of choice, you can subject them to clone scale-up (to proceed to **Clone Scale-Up**, see page 41).

Clone scale-Up

Introduction

After isolating your clones of interest (previous section), transfer single-cell colonies from 96-well plates to 24-well plates, and then scale up the volume of cells every 3–7 days by transferring each clone into the next larger plate or vessel (i.e., 24-well plates to 6-well plates to T-25 flask and then to 125-mL shaker flasks).

Note

The total clone scale-up process from a 96-well plate to a 125-mL flask takes about 2–4 weeks, depending on the growth rate of each clone. Monitor the protein production of each clone using your method of choice (see page 25) and carry over the top-producing clones to the next stage in the scale-up process.

Materials needed

- Single cell-derived clones in 96-well plates
- CD OptiCHO™ Medium supplemented with 6 mM L-glutamine
- Sterile tissue culture dishes (24-well and 6-well), sterile T-25 flasks, and sterile 125-mL polycarbonate shaker flasks
- Non-shaking incubator set at 37°C, humidified atmosphere at **5% CO_2**
- Shaking incubator set at 37°C, humidified atmosphere at **8% CO_2**, shaking at 130–135 rpm
- Assay for determining protein production

Protocol

1. Perform clone expansion with an appropriate number of top-producing clones at any stage during screening (i.e., 96-well plate, 24-well plate, or 6-well plate stage).
2. When individual clones are 20–100% confluent (10–14 days) in 96-well plates, aseptically harvest the desired clones by pipetting up and down gently and transferring the entire content of each well into a separate well of 24-well tissue culture plates containing 0.5–1 mL of fresh growth medium (CD OptiCHO™ supplemented with 6 mM L-glutamine).
3. Continue expansion; after 3–5 days, transfer the desired clones into the next larger culture plate or vessel using the same procedure. The final culture volumes in 6-well plates and T-25 flasks are 2–3 mL and 5–7 mL, respectively
4. Once the clones are expanded to 125-mL shaker flasks, incubate the cells at 37°C and 8% CO_2, with shaking at 130–135 rpm.

IMPORTANT!

Prepare frozen cell stocks prior to optimizing protein production. You may then optimize protein production using different culture conditions, or continue the scale-up procedure to meet your bioproduction needs. To ensure the stability of the screened clones, do not use selection reagents such as Geneticin® Selective Antibiotic or MTX during the scale-up process.

Methods for single-subunit protein expression

One-page flowcharts

Introduction

The following pages contain flowcharts to aid you in your expression experiments. Each flowchart consists of an outline of the necessary steps and space for notes that you can then transfer to your laboratory notebook.

Using the one-page flowcharts

The page numbers by the experimental steps in the flowcharts are hyperlinked to detailed protocols. Hold down the CTRL key as you click on the page number given in the flowchart to access the appropriate detailed protocol. You may also print the individual flowcharts for use in the laboratory.

Note

The one-page protocols are experimental guidelines only; they do **not** provide detailed protocols to successfully perform the steps necessary for expressing your protein of interest. Note that the times shown for various experimental steps are approximations; the actual times depend on your protein of interest and the specific workflows you choose.

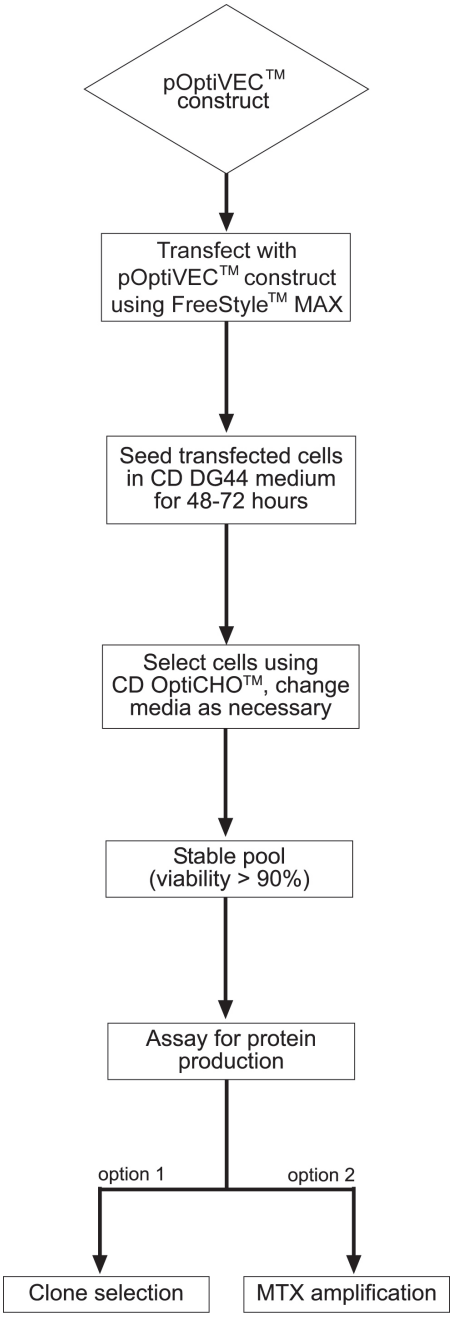
We recommend that you familiarize yourself with the detailed protocols before starting your experiments.

Continued on next page

Expressing single-subunit protein using pOptiVEC™ expression construct

Introduction

The flowchart below depicts the major steps to transfect suspension CHO DG44 Cells (cGMP banked) with the pOptiVEC™ construct containing your gene of interest (GOI) using the FreeStyle™ MAX Reagent.

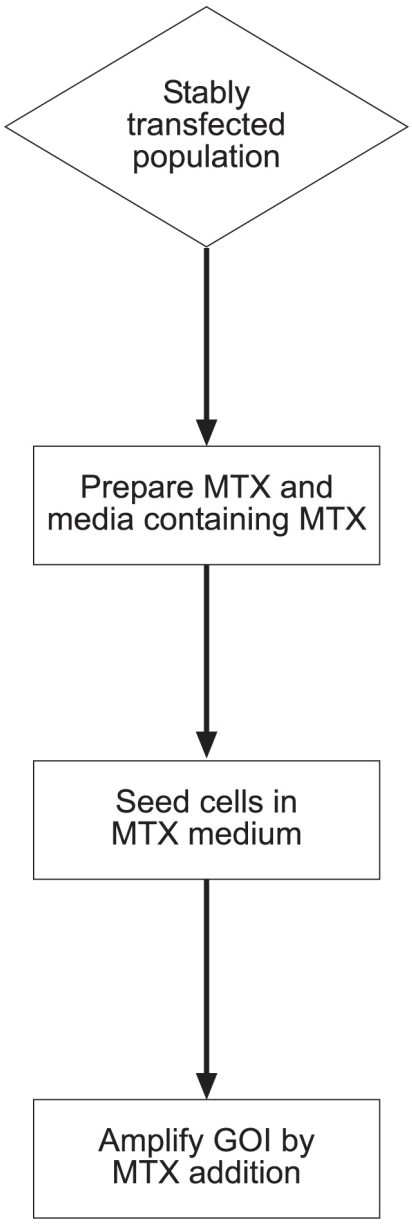

pOptiVEC™ construct
Transfect with pOptiVEC™ construct using FreeStyle™ MAX
Seed transfected cells in CD DG44 medium for 48-72 hours
Select cells using CD OptiCHO™, change media as necessary
Stable pool (viability > 90%)
Assay for protein production
option 1
option 2
Clone selection
MTX amplification

Page	Time Line	Notes
page 46	Transfection: 1 day	
page 52	2–3 days	
page 52	Selection: 12–14 days	
page 53	7–10 days	
Clone selection : page 57		
MTX amplifi-cation : page 55		

Genomic amplification by MTX addition

Introduction

The flowchart below depicts the major steps to amplify the copy number of your gene of interest (GOI) using MTX (as methotrexate hydrate) for increased production of the protein of interest.

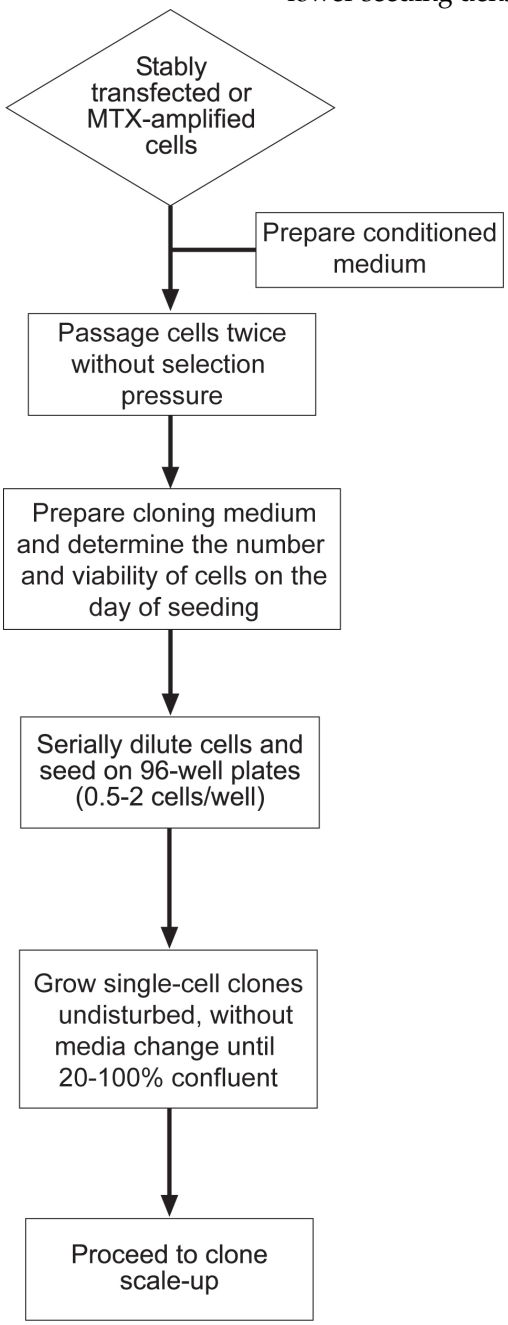

Stably transfected population
Prepare MTX and media containing MTX
Seed cells in MTX medium
Amplify GOI by MTX addition

	Page	Time Line	Notes
Prepare MTX and media containing MTX	page 55	Media prep: 1–3 hours	
Seed cells in MTX medium	page 56	Seeding cells: 1 hour	
Amplify GOI by MTX addition	page 56	2–4 weeks (variable depending on protein of interest)	

Clonal selection by limiting dilution (single-subunit protein)

Introduction

The flowchart below depicts the major steps to obtain a clonal cell line (i.e., derived from a single cell) for the production of a single-subunit protein by diluting the pool of stably transfected cells or the MTX-amplified cells to 0.5–2 cells per well in a 96-well plate containing cloning medium. In most cases, one of the cells forms a distinct colony that can later be scaled up. You may also statistically calculate the desired number of cells per well to help ensure monoclonality. However, using a lower seeding density may result in decreased cloning efficiency.


Stably transfected or MTX-amplified cells
Prepare conditioned medium
Passage cells twice without selection pressure
Prepare cloning medium and determine the number and viability of cells on the day of seeding
Serially dilute cells and seed on 96-well plates (0.5-2 cells/well)
Grow single-cell clones undisturbed, without media change until 20-100% confluent
Proceed to clone scale-up

	Page	Time Line	Notes
Prepare conditioned medium	page 57	3–5 days	
Passage cells twice without selection pressure		5–7 days	
Prepare cloning medium and determine the number and viability of cells on the day of seeding	page 58–59	2 hours	
Serially dilute cells and seed on 96-well plates (0.5-2 cells/well)	page 59	4 hours	
Grow single-cell clones undisturbed, without media change until 20-100% confluent	page 60	10–14 days	
Proceed to clone scale-up	page 61		

Transfecting CHO DG44 Cells with FreeStyle™ MAX Reagent for single-subunit protein expression

Introduction

You will use FreeStyle™ MAX Reagent to transfect suspension CHO DG44 Cells (cGMP banked) with the pOptiVEC™ expression construct containing your gene of interest

IMPORTANT!

If you are expressing a single-subunit protein, you **must** generate your expression construct using the pOptiVEC™-TOPO® TA vector. The pOptiVEC™ expression construct allows genomic amplification by MTX amplification and clonal selection by limiting dilution in cloning medium as recommended on page 57.

Do not use an expression construct generated with the pcDNA™3.3 TOPO® TA vector, because the CD-OptiCHO™ Medium is incompatible with pcDNA™3.3 expression constructs.

Plasmid preparation

The pOptiVEC™ plasmid construct must be clean, sterile, and free from contamination with phenol and sodium chloride for transfection into CHO DG44 Cells (cGMP banked). Contaminants may kill the cells, and salt interferes with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using an endotoxin-free or a low-endotoxin kit such as the PureLink® HiPure Plasmid Midiprep DNA Kit (see page 71 for ordering information).

Linearizing the plasmids

Prior to using the Freedom™ DG44 Kit to transfect CHO DG44 Cells (cGMP banked) with your pOptiVEC™ construct containing your gene of interest, you may linearize the plasmid. Linearizing your vector may not improve transfection efficiency, but it increases the chance that the vector will integrate into the host cell genome without disrupting the gene of interest or other elements required for expression in mammalian cells.

- We suggest using *Pvu* I, which cuts once in the ampicillin resistance gene on the plasmid. Other unique restriction sites are possible. Complete restriction map of pOptiVEC™-TOPO® TA is available at **www.lifetechnologies.com**. **Be sure that your insert does not contain the restriction enzyme site you use to linearize the vector.**

  Note: If an appropriate linearization site is not present, you may transfect the circular plasmid. Transfection efficiency will not be affected.
- After digestion, precipitate the DNA, resuspend pellet in sterile water, and re-quantify using your method of choice.

Continued on next page

Transfecting CHO DG44 Cells with FreeStyle™ MAX Reagent for single-subunit protein expression, continued

Recommendation

Calculate the number of CHO DG44 Cells (cGMP banked) that you will need for your transfection experiment and expand cells accordingly. Make sure that the cells are healthy and greater than 95% viable before proceeding to transfection.

Materials needed

- Suspension CHO DG44 Cells (cGMP banked) cultured in complete CD DG44 Medium at 5×10^5 viable cells/mL
- Purified, linearized pOptiVEC™ plasmid DNA containing your gene of interest, prepared as explained on page 46
- FreeStyle™ MAX Reagent (supplied with the kit; store at 2°C to 8°C until use)
- OptiPRO™ SFM (supplied with the kit; pre-warmed to room temperature)
- Disposable, sterile, 125-mL polycarbonate Erlenmeyer flasks
- Orbital shaker in 37°C incubator with a humidified atmosphere of 8% CO_2
- Reagents and equipment to determine viable and total cell counts (e.g., Trypan Blue, hemacytometer, or an automated cell counter such as the Countess® Automated Cell Counter)

Optimal transfection conditions

To transfect suspension CHO DG44 Cells (cGMP banked) in a 30 mL volume, we recommend using the following optimized conditions:

- **Final transfection volume:** 30 mL
- **Number of cells to transfect:** total of 1.5×10^7 viable cells (cell density at time of transfection should be 5×10^5 viable cells/mL)
- **Amount of plasmid DNA:** 18 µg
- **FreeStyle™ MAX Reagent:** 15 µL

Note: Further optimization of culture volume or transfection conditions is not necessary for stable cell line production.

Continued on next page

Transfecting CHO DG44 Cells with FreeStyle™ MAX Reagent for single-subunit protein expression, continued

Transfection procedure using pOptiVEC™ expression construct

Follow the procedure below to transfect CHO DG44 Cells (cGMP banked) in a **30-mL volume** using your pOptiVEC™ expression construct. We recommend including negative controls (no DNA, no FreeStyle™ MAX Reagent) in your experiment to help you evaluate your results.

1. At 48 hours before transfection, pass CHO DG44 Cells (cGMP banked) at 3×10^5 cells/mL in complete CD DG44 Medium. Place the flask(s) on an orbital shaker platform rotating at 130–135 rpm at 37°C, 8% CO_2.
2. At 24 hours before transfection, pass CHO DG44 Cells (cGMP banked) at 3×10^5 cells/mL in complete CD DG44 Medium. Place the flask(s) on an orbital shaker platform rotating at 130–135 rpm at 37°C, 8% CO_2.
3. On the day of transfection, perform a viable cell count (see page 16). To ensure optimal transfection results, viability of cells must be over 95%.
4. For each transfection or control, transfer 1.5×10^7 viable CHO DG44 Cells (cGMP banked) to a new 125-mL flask. Add pre-warmed, complete CD DG44 Medium to a final volume of 30 mL. Place flask in shaker until ready to transfect.

   Note: Do not centrifuge cells prior to transfection to avoid a decrease in transfection efficiency.
5. Gently invert the tube of FreeStyle™ MAX Reagent several times to mix. **Do not vortex**.
6. Add 18 µg of plasmid DNA to 600 µL OptiPRO™ SFM and mix gently. Add 15 µL of FreeStyle™ MAX Reagent into 600 µL OptiPRO™ SFM and mix gently.
7. Immediately add diluted FreeStyle™ MAX Reagent solution to the diluted DNA solution and mix gently. Incubate the DNA-FreeStyle™ MAX mix for 10 minutes at room temperature to allow complexes to form. **Do not incubate for longer than 20 minutes**.
8. Drop wise add 1.2 mL of DNA-FreeStyle™ MAX Reagent complex into the 125-mL flask containing cells while slowly swirling the flask.
9. Incubate transfected cell cultures at 37°C, 8% CO_2 on an orbital shaker platform rotating at 130–135 rpm.
10. At 48 hours post transfection, pass cells into HT-deficient, complete CD OptiCHO™ Medium (see page 13). Proceed to **Selecting Stable Transfectants for Single-subunit Protein Expression**, page 52.

Transfecting CHO DG44 Cells with Neon® for single-subunit protein expression (optional)

Recommendation

Calculate the number of CHO DG44 Cells (cGMP banked) that you will need for your transfection experiment and expand cells accordingly. Make sure that the cells are healthy and greater than 95% viable before proceeding to transfection.

Materials needed

- Suspension CHO DG44 Cells (cGMP banked) cultured in complete CD DG44 Medium
- Purified, linearized pOptiVEC™ plasmid DNA containing your gene of interest, as determined on page 25
- Neon® Transfection system (see page 71 for ordering information)

  Note: Both Resuspension Buffer R and Buffer E2 used in this protocol are Animal Origin-Free (AOF).
- Disposable, sterile T-75 tissue culture flasks
- A static culture incubator at 37°C with a humidified atmosphere of 8% CO_2
- Reagents and equipment to determine viable and total cell counts (e.g., Trypan Blue, hemacytometer, or an automated cell counter such as the Countess® Automated Cell Counter)

Optimal transfection conditions

To transfect suspension CHO DG44 Cells (cGMP banked) in a 15–20 mL volume, we recommend using the following optimized conditions:

- **Number of cells to transfect:** total of 1.0×10^7 viable cells (in 100 µL Neon® tip)
- **Amount of each plasmid DNA:** 10 µg each (total 20 µg)
- **Neon® Electroporation program:** #5, #9 or #24

  Note: We observed equivalent transfection efficiency (≥ 85%) all of these three programs at 24 hours and 48 hours post-transfection. We recommend using these programs as a starting point to test the stable transfection of your gene of interest.

General guidelines of transfection

- Prepare high-quality plasmid DNA at a concentration of 1–5 µg/µL in deionized water or TE buffer.
- Use an appropriate GFP plasmid transfected in parallel to determine transfection efficiency if possible.
- Discard the Neon® Tips after two uses and Neon® Tubes after 10 uses as a biological hazard. Change tube and buffer when switching to a different plasmid DNA or cell type.
- The plasmid DNA amount should not exceed 10% of total volume used for transfection.
- Prepare extra volume of cell suspension to ensure that the desired volume is available at the time of transfection.

Continued on next page

Transfecting CHO DG44 Cells with Neon® for single-subunit protein expression, continued

Preparing cells

1. 24 hours before transfection, passage CHO DG44 cells at 3×10^5 cells/mL in complete CD DG44 medium. Place the flask(s) on an orbital shaker platform rotating at 130–135 rpm at 37°C, 80% relative humidity, and 8% CO_2
2. On the day of transfection, harvest appropriate amount of cells (total of 1.0×10^7 viable cells per transfection) and wash the cells in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}.
3. Resuspend the cell pellet in appropriate volume (100 µL per transfection) of Resuspension Buffer R (included with Neon® Kits) at a final density of 1.0×10^8 cells/mL.
4. Prepare T-75 flask(s) by filling the flask(s) with 15–20 mL of complete CD DG44 medium without antibiotics and pre-incubate flask(s) at 37°C in an 80% relative humidity, 8% CO_2 incubator for at least 15 minutes.

Transfection procedure for expression of single-subunit protein

1. Fill the Neon® Tube with 3 mL of Electrolytic Buffer E2 (for a 100 µL Neon® tip).
2. Insert the Neon® Tube into the Neon® Pipette Station until you hear a click.
3. Transfer the appropriate amount of plasmid DNA (20 µg DNA per transfection) into a sterile, 1.5 mL microcentrifuge tube.
4. Add appropriate amount of cells (100 µL per transfection) to the tube containing plasmid DNA and gently mix.
5. Press the push-button on the Neon® Pipette to the second stop to open the clamp and insert the top-head of the Neon® Pipette into the Neon® Tip until the clamp fully picks up the mounting stem of the piston. Gently release the push-button, continuing to apply a downward pressure on the pipette, ensuring that the tip is sealed onto the pipette without any gaps.
6. Press the push-button on the Neon® Pipette to the first stop and immerse the Neon® Tip into the cell-DNA mixture. Slowly release the push-button on the pipette to aspirate the cell-DNA mixture into the Neon® Tip.
7. Insert the Neon® Pipette with the sample vertically into the Neon® Tube placed in the Neon® Pipette Station until you hear a click.
8. Ensure that you have selected the appropriate electroporation protocol and press **Start** on the touchscreen.

   The Neon® device automatically checks for the proper insertion of the Neon® Tube and Neon® Pipette before delivering the electric pulse.

   The touchscreen displays "Complete" to indicate that electroporation is complete.

Continued on next page

Transfection procedure for expression of single-subunit protein, continued

9. Remove the Neon® Pipette from the Neon® Pipette Station and immediately transfer the samples from the Neon® Tip by pressing the push-button on the pipette to the first stop into the prepared T-75 flask(s) containing pre-warmed medium without antibiotics. Discard the Neon® Tip into an appropriate biological hazardous waste container.
10. Repeat Steps 5–11 for the remaining samples. Change the Neon® Tips after two uses and Neon® Tubes after ten uses. Use a new Neon® Tip and Neon® Tube for each new plasmid DNA or cell type.
11. Gently rock the T-flask to assure even distribution of the cells. Incubate the flask at 37°C in a humidified CO_2 incubator.
12. If you are not using the Neon® device, turn the power switch on the rear to "OFF".
13. 48 hours post transfection, centrifuge the cells at 300 × *g* for 5 minutes and remove the medium by aspiration. Resuspend cells with 30 mL of pre-warmed complete CD OptiCHO™ Medium containing 500 µg/mL Geneticin® Selective Antibiotic to give a final cell density of 5×10^5 viable cells/mL in a 125-mL shaker flask. Proceed to **Selecting Stable Transfectants for Single-subunit Protein Expression**, next page.

Selecting stable transfectants for single-subunit protein expression

Introduction

To obtain cell lines that produce high levels of your protein, first select for a pool of stably-transfected cells, in which the linearized pOptiVEC™ construct has integrated into the host cell genome. Since CHO DG44 Cells (cGMP banked) are DHFR-deficient and require supplementary hypoxanthine and thymidine (HT), only cells that have been transfected with your pOptiVEC™ expression construct can be propagated in CD OptiCHO™ Medium.

DHFR

DHFR catalyzes the reduction of 5, 6-dihydrofolate to 5, 6, 7, 8-tetrahydrofolate, which is essential for DNA synthesis. CHO-derived DG44 Cells lack DHFR activity and must be propagated in medium containing the purine precursors hypoxanthine and thymidine (HT) unless the cells are stably transfected with a vector that expresses DHFR.

DHFR also functions as a genomic amplification marker for your gene of interest using methotrexate (MTX) selection (Kaufman *et al.*, 1985; Tanaka *et al.*, 2002). See page 55 for more details on genomic amplification using MTX.

Selecting stable transfectants in CD OptiCHO™ Medium

Transfected CHO DG44 Cells should be passaged in complete CD OptiCHO™ Medium for selection. To passage cells:

1. Determine viable and total cell counts using your preferred method (see page 16).
2. Dilute the cells in pre-warmed complete CD OptiCHO™ Medium to give a final cell density of 5×10^5 viable cells/mL.
3. Incubate flasks in a 37°C incubator containing a humidified atmosphere of 8% CO_2 on an orbital shaker platform rotating at 130–135 rpm.
4. Centrifuge cells at 300 × *g* for 5 minutes, remove the medium by aspiration, and add fresh medium to the desired final volume every 3–4 days for 10–14 days until cell viability increases to >90% (see **Note** below). It is not necessary to centrifuge the cells and re-suspend them in complete fresh medium if the dilution factor at the time of passage is >2.
5. When culture reaches >90% viability, maintain at 3×10^5 viable cells/mL and scale up the culture as needed.

Note

During the selection rounds, cell viability may drop dramatically (to <10%) due to the death of untransfected and transiently-transfected cells. To promote optimal growth of stably transfected cells, maintain cultures as described in Steps 4–5.

Assessing productivity

Protein production

To check for production of your protein during stable cell establishment, you may take an aliquot of growth media and perform SDS-PAGE, protein-specific ELISA, or the bioactivity assay of choice to determine that your cells are producing your protein of interest.

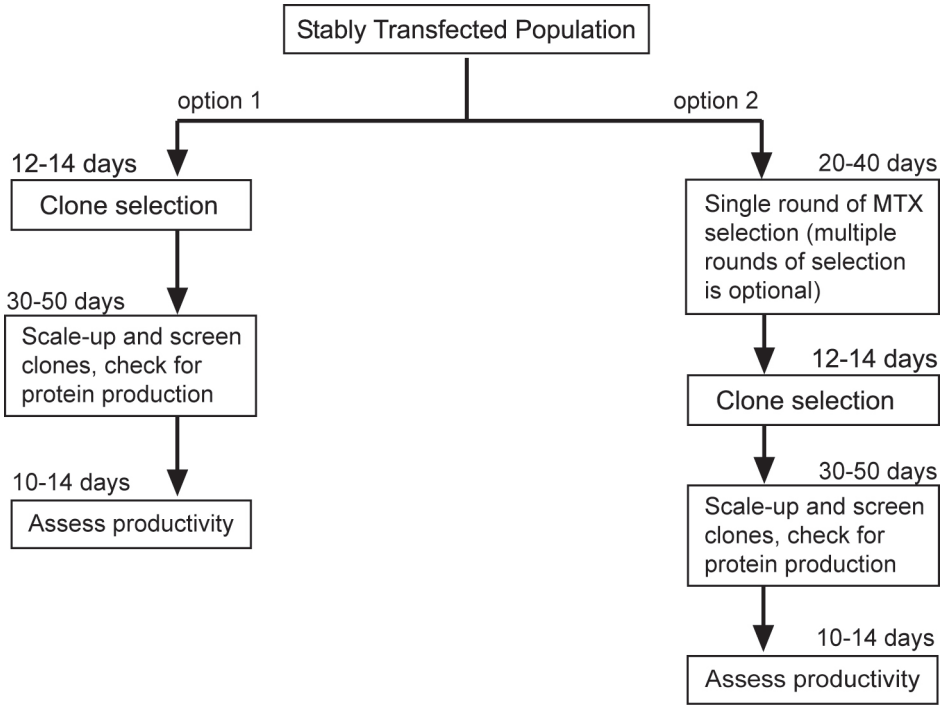
IMPORTANT!

When you have a pool of stably-transfected cells, freeze several aliquots of the pool using the procedure on page 18.

Choosing a workflow

At this stage you will have a population of stably-transfected CHO DG44 Cells expressing your protein of interest at various levels. For most bioproduction applications, several clonally-derived cell lines producing your protein are desirable for screening. However, the productivity of each clone depends upon the integration locus of the plasmid(s), the response to amplification using MTX, and the nature of the protein.

Depending on your protein production needs, the time and effort required to generate clonal, high-producing cell lines also vary. Several common pathways from stable pool to clone scale-up are outlined below. **Note that the times shown for various experimental steps are approximations, and the actual times will depend on your protein of interest and the specific workflows you choose.**



Continued on next page

Assessing productivity, continued

Points to consider

When choosing a workflow, consider the amount of protein you wish to produce, your available resources, and the amount of time it will take to obtain your clonal, high-producing cell lines. Because MTX amplification produces a polyclonal population, you must always perform clone selection prior to scale-up.

Additional cloning media, supplements, and other products may be purchased separately (page 70).

Assessing productivity

The following protocol is used to assess the productivity in batch culture. Nutrition feed protocol can be added appropriately if needed.

1. Seed fully recovered cells (viability >90%) at 3×10^5 viable cells/mL with 30 mL fresh medium (CD OptiCHO™ media supplemented with 4 mM L-glutamine) in 125-mL shaker flasks. The culture volume can be scaled up based on the schedule of sampling.

   Note: You can adjust the L-glutamine concentration as needed (for example 0–8 mM).

2. Sample cultures daily or at regular intervals (for example, on Day 0, 3, 5, 7, 10, 12, and 14) until culture viability drops below 50% (whichever comes first) to determine the cell density, viability, and productivity.

   Keep the cell seed-train growing according to the subculture protocol until you successfully complete your productivity study.

Next steps

- To perform 1 round of genomic amplification using MTX selection to obtain a population of cells expressing high levels of your protein, see page 55.
- To perform limiting dilution cloning to obtain single clones expressing high levels of your protein, see page 57.
- To scale up your clones for protein expression, see page 61.

Genomic amplification by MTX addition

Introduction

Methotrexate (MTX) is a folic acid antagonist that is actively transported into cells by the folate transporter. In the cell, it is converted to a high molecular weight polyglutamate metabolite by folylpolyglutamate synthase, which binds to DHFR and inhibits its activity.

If MTX is present in the medium, cells compensate by increasing the DHFR copy number in the genome to overcome inhibition by MTX. Since the gene of interest is integrated into the same genetic locus as DHFR, the gene of interest is amplified as well, leading to increased production of the protein of interest (Kaufman *et al.*, 1985; Tanaka *et al.*, 2002). MTX (as methotrexate hydrate) is available from Sigma (10 mg, Sigma Cat. no. A6770).

CAUTION!

MTX is toxic to the skin, eyes, and respiratory system. Wear suitable protective clothing, gloves, and eye and face protection when working with MTX. Refer to the product MSDS for complete precautions.

Preparing 1 mM MTX

To prepare a 1 mM MTX stock solution:

1. Dissolve 10 mg MTX in 22 mL of PBS.
2. Filter-sterilize the solution through a 0.22 µm filter.
3. Store in 250 µL aliquots at –20°C.

Preparing media with MTX

- To make complete CD OptiCHO™ Medium containing MTX, use complete CD OptiCHO™ Medium (prepared to contain L-glutamine) plus the required concentration of MTX.
- Do **not** add Geneticin® reagent to the complete CD OptiCHO™ Medium as the cells transfected with only the pOptiVEC™ construct do **not** express resistance to Geneticin® Selective Antibiotic.
- Using the sterile, 1 mM MTX stock solution (prepared as described above), prepare media containing the appropriate concentration of MTX.

Continued on next page

Genomic amplification by MTX addition, continued

One round of MTX amplification

The productivity of each clone depends upon the integration locus of your expression construct, the response to amplification using MTX, and the nature of your protein. Depending on your protein production needs, your available time, and your resources, you may perform one round of MTX amplification at various concentrations (such as 50 nM, 100 nM, 250 nM, 500 nM, and 1 µM). Additional rounds of MTX amplification may be performed using higher concentrations (such as 2 µM and 4 µM) to potentially increase your protein production.

Protocol for MTX amplification

1. For each cell pool, centrifuge cells at 300 × *g* for 5 minutes, then aspirate old medium.
2. Seed cells at a density of 3×10^5 viable cells/mL in 100–300 mL of media containing various concentrations of MTX in 0.5–1 liter shaker flasks.
3. Incubate flasks at 37°C/8% CO_2 with shaking at 130–135 rpm.
4. Passage cells into fresh medium containing MTX at 2×10^5–3×10^5 viable cells/mL in shaker flasks every 3 or 4 days. Spin down the cells and re-suspend them in fresh medium if the dilution factor at passage is < 2.
5. Passage cells at 2×10^5 to 3×10^5 viable cells/mL when the viability starts increasing. The cells amplified with low concentration of MTX recover faster than that with high concentration of MTX.
6. When cell viability is >90%, freeze cells and start protein productivity analysis.

Next steps

Because MTX amplification produces a polyclonal population of cells, you must always perform clone selection (page 57) prior to clone scale-up (page 61).

Clonal selection by limiting dilution

Introduction

Development of a CHO cell line for commercial production of a recombinant protein requires clonality of the final cell population, achieved by limiting dilution cloning (LDC). Before performing LDC, expand the stably transfected or MTX-amplified cells in CD OptiCHO™ medium supplemented with 8 mM glutamine and without any selection pressure for at least two passages. On the day of cloning, dilute the cells to seed 0.5–2 cells per well in a 96-well plate. In most cases, one of the cells forms a distinct colony that can be scaled up using the procedure described below. You may also statistically calculate the desired number of cells per well to help ensure clonality of the colonies. However, using a lower seeding density may result in decreased cloning efficiency.

Cloning medium considerations

- Because the growth rate and protein production of each clone vary, you may need to optimize clonal selection conditions by adjusting the number of cells per well, the volume of the cloning medium, and various media supplements.
- CHO DG44 transfected pools achieve good cloning efficiency when CD FortiCHO™ Medium is used as the cloning medium.
- To achieve the highest cloning efficiency in LDC, CHO DG44 transfected pools require the addition of conditioned media and HT Supplement. See page 71 for ordering information.
- Depending on how many clones you wish to screen before scale-up, you may increase the number of limiting dilution plates according to your anticipated cloning efficiency and level of protein production. The number of clones obtained from a 96-well plate varies, depending on the experiment.

Preparing conditioned medium

You can collect spent medium from a transfected CHO DG44 pool and use it as conditioned medium. We recommend using DG44 cells transfected with an empty pOptiVEC™ vector, which you can generate by inserting a small PCR fragment to close the TOPO cloning site. The advantage of this method is that you can use the conditioned medium for cloning with any transfected parental DG44 pool. Freeze the transfected cell pool and use as needed to generate conditioned medium.

1. Seed 3×10^5 viable cells/mL in a sufficient culture volume of CD OptiCHO™ medium supplemented with 8 mM glutamine to generate enough conditioned medium needed for cloning experiments.
2. Grow the cells in batch culture for 5 days.
3. Centrifuge the cultures and collect the supernatant (spent medium). Discard the cell pellet.
4. Sterilize the collected spent medium by membrane filtration.
5. Freeze the condition medium in aliquots at –80°C until needed.

Continued on next page

Clonal selection by limiting dilution, continued

Preparing cloning medium

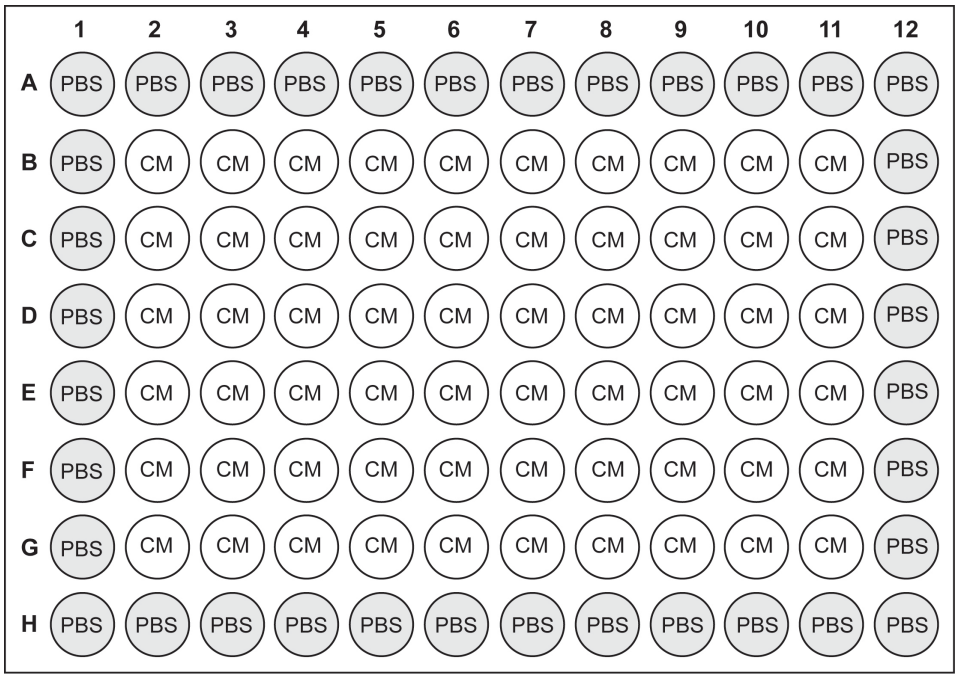
The procedure described below uses CD FortiCHO™ Medium as the cloning medium. One 50-mL conical tube is sufficient to seed approximately 200 wells at 1 cell/well. For greater numbers of wells or plates, adjust the volumes accordingly.

1. Thaw L-glutamine to be used in preparation of completed cloning medium.
2. For each 100 mL of completed cloning medium required, aseptically mix the following:

   86 mL of basal CD FortiCHO™ Medium
   3 mL of freshly thawed 200 mM L-glutamine
   10 mL of conditioned medium
   1 mL of 100X HT supplement
3. For each 40 mL of cells required for plating, add 39.8 mL of completed cloning medium to a 50-mL centrifuge tube and mix by gentle inversion 5–6 times.
4. Pre-warm the medium at 37°C for one hour before using it for plating the cells (page 60).

Plate setup

1. Label a sufficient number of 96 well plates for your procedure.
2. Add 200 µL/well of sterile PBS to all peripheral wells to avoid evaporation during incubation (as shown in the figure below).


1 2 3 4 5 6 7 8 9 10 11 12
A PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS
B PBS CM CM CM CM CM CM CM CM CM CM PBS
C PBS CM CM CM CM CM CM CM CM CM CM PBS
D PBS CM CM CM CM CM CM CM CM CM CM PBS
E PBS CM CM CM CM CM CM CM CM CM CM PBS
F PBS CM CM CM CM CM CM CM CM CM CM PBS
G PBS CM CM CM CM CM CM CM CM CM CM PBS
H PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS

CM = cells diluted in **cl**oning **m**edium

Continued on next page

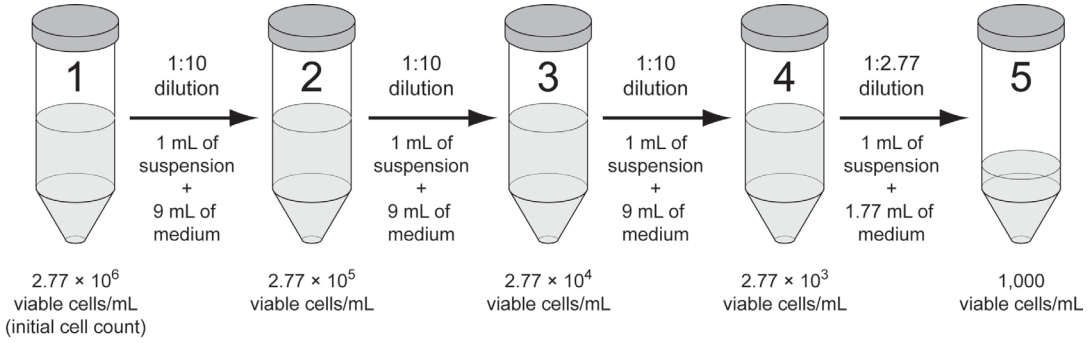
Clonal selection by limiting dilution, continued

Cell counting and dilution

1. Label five 50-mL conical tubes "1" through "5".
2. Pipette 5 to 10 mL of your transfected pool CHO DG44 cells into the 50-mL tube labeled "1".
3. Determine the viable cells/mL accurately using your method of choice.
4. Serially dilute the cells to a final concentration of 1000 cells/mL using growth medium (CD OptiCHO™ Medium supplemented with 8 mM L-glutamine) as shown in figure below to yield a seeding density of 1 cell/well. If a different cell/well seeding density is desired, adjust accordingly in the final dilution step.

   Note: Mix cells gently after each dilution by inverting the capped tube 5 to 6 times. Avoid foaming.

Example of cells serially diluted in CD OptiCHO™ Medium with an initial count of 2.77×10^6 viable cells/mL


1
1:10 dilution
1 mL of suspension + 9 mL of medium
2
1:10 dilution
1 mL of suspension + 9 mL of medium
3
1:10 dilution
1 mL of suspension + 9 mL of medium
4
1:2.77 dilution
1 mL of suspension + 1.77 mL of medium
5
2.77 × 10^6 viable cells/mL (initial cell count)
2.77 × 10^5 viable cells/mL
2.77 × 10^4 viable cells/mL
2.77 × 10^3 viable cells/mL
1,000 viable cells/mL

Continued on next page

Clonal selection by limiting dilution, continued

Plating cells

1. After warming the cloning medium (step 4, page 58), remove it from the incubator and place it in the laminar flow hood.
2. Pipette 0.2 mL of the cell suspension from "Tube 5" (1000 cells/mL) into the cloning medium.
3. Mix the cell suspension gently by inverting the tube 5 or 6 times and transfer it into a sterile reagent reservoir or trough.
4. Aseptically dispense 200 µL of the diluted cells into each of the empty 60 wells of each 96-well plate, using a multi-channel pipettor.
5. Incubate the plates **undisturbed** for 10–14 days at 37°C and 5% CO_2 in humidified air in a static (non-shaking) incubator. Stack no more than 5 plates together.
6. After day 10 of incubation, examine the wells visually using a microscope for growth of monoclonal colonies.
7. Perform your primary screen (i.e., protein assay of choice) to determine the cloning efficiency and identify clones of interest.
8. Calculate the percent cloning efficiency, normalized at 1 cell per well, as follows

$$\text{Cloning Efficiency} = \frac{\text{Number of wells showing growth of a single colony}}{\text{Number of wells seeded} \times \text{cells per well seeded}} \times 100\%$$

For example, the cloning efficiency with an experiment of 120 single colonies growing out of total 600 seeded wells (10 plates with 60 seeded wells per plate) at a seeding density of 0.5 cell per well is 40%.

Next steps

After testing these clones for high levels of protein production using your method of choice, you can subject them to clone scale-up (to proceed to **Clone scale-up**, see page 61).

Clone scale-up

Introduction

After isolating your clones of interest (previous section), transfer single-cell colonies from 96-well plates to 24-well plates, and then scale up the volume of cells every 3–7 days by transferring each clone into the next larger plate or vessel (i.e., 24-well plates to 6-well plates to T-25 flask and then to 125-mL shaker flasks).

Note

The total clone scale-up process from a 96-well plate to a 125-mL flask takes about 2–4 weeks, depending on the growth rate of each clone. Monitor the protein production of each clone using your method of choice (see page 53) and carry over the top-producing clones to the next stage in the scale-up process.

Materials needed

- Single cell-derived clones in 96-well plates
- CD OptiCHO™ Medium supplemented with 6 mM L-glutamine
- Sterile tissue culture dishes (24-well and 6-well), sterile T-25 flasks, and sterile 125-mL polycarbonate shaker flasks
- Non-shaking incubator set at 37°C, humidified atmosphere at **5% CO_2**
- Shaking incubator set at 37°C, humidified atmosphere at **8% CO_2**, shaking at 130–135 rpm
- Assay for determining protein production

Protocol

1. Perform clone expansion with an appropriate number of top-producing clones at any stage during screening (i.e., 96-well plate, 24-well plate, or 6-well plate stage).
2. When individual clones are 20–100% confluent (10–14 days) in 96-well plates, aseptically harvest the desired clones by pipetting up and down gently and transferring the entire content of each well into a separate well of 24-well tissue culture plates containing 0.5–1 mL of fresh growth medium (CD OptiCHO™ Medium supplemented with 6 mM L-glutamine).
3. Continue expansion; after 3–5 days, transfer the desired clones into the next larger culture plate or vessel using the same procedure. The final culture volumes in 6-well plates and T-25 flasks are 2–3 mL and 5–7 mL, respectively.
4. Once the clones are expanded to 125-mL shaker flasks, incubate the cells at 37°C and 8% CO_2, with shaking at 130–135 rpm.

IMPORTANT!

Prepare frozen cell stocks prior to optimizing protein production. You may then optimize protein production using different culture conditions, or continue the scale-up procedure to meet your bioproduction needs. To ensure the stability of the screened clones, do not use selection reagents such as Geneticin® Selective Antibiotic or MTX during the scale-up process.

Appendix A: Troubleshooting

Troubleshooting

Culturing CHO DG44 Cells (cGMP banked)

The table below lists some potential problems and possible solutions that may help you troubleshoot your cell culture experiment.

Observation	Reason	Solution
No viable cells after thawing original vial	Cells not stored correctly	Order new cell stock and store in liquid nitrogen. Keep in liquid nitrogen until thawing.
	Incorrect thawing medium or method	• Use pre-warmed CD DG44 Medium supplemented with 8 mM L-glutamine and 18 mL/L Pluronic® F-68. **Do not use** CD OptiCHO™ Medium to propagate DHFR-negative CHO DG44 Cells (cGMP banked). • Do not add antibiotics to media as this may negatively impact cell growth. • Incubate cultures on an orbital shaker set at 130–135 rpm in a 37°C incubator with a humidified atmosphere of 8% CO_2.
No viable cells after thawing stocks	Cells not frozen correctly	Follow the protocol on page 18 to freeze cells.
	Incorrect thawing medium	• Use pre-warmed CD DG44 Medium supplemented with 8 mM L-glutamine and 18 mL/L Pluronic® F-68. **Do not use** CD OptiCHO™ Medium to propagate DHFR-negative CHO DG44 Cells (cGMP banked). • Do not add antibiotics to media as this may negatively impact cell growth. • Incubate cultures on an orbital shaker set at 130–135 rpm in a 37°C incubator with a humidified atmosphere of 8% CO_2.

Continued on next page

Culturing CHO DG44 Cells (cGMP banked)

The table below lists some potential problems and possible solutions that may help you troubleshoot your cell culture experiment.

Observation	Reason	Solution
Cells grow slowly	Incorrect growth medium	• Use pre-warmed CD DG44 Medium supplemented with 8 mM L-glutamine and 18 mL/L Pluronic® F-68. • Do not use CD OptiCHO™ Medium to propagate DHFR-negative CHO DG44 Cells (cGMP banked).
	Shaker not set up properly	Shake on an orbital shaker at 130–135 rpm in 37°C incubator with a humidified atmosphere of 8% CO_2.
	Medium is foamy	• Keep shaker speed at 130–135 rpm. • At larger culture scales, you may add FoamAway™ Irradiated AOF to prevent foaming (see page 71).
	Cells too old	Use healthy CHO DG44 Cells (cGMP banked) under generation 25; do not overgrow.
	Cell culture clumpy	• Provide agitation of the culture, a regular and frequent cell passage schedule, and maintenance of cells at recommended densities. • Use Anti-Clumping Agent (see page 71 for ordering information). • **Do not** use anti-clumping agent during transfection. Anti-clumping agent needs to be removed 2 passages before transfection.
	Cells overheat	• Calibrate the incubator by comparing the actual temperature of the medium in a culture flask while shaking on the platform to the temperature setting of the incubator. Use a flask containing growth medium, but no cells. Then adjust the incubator setting to the defined temperature.

Continued on next page

Transfection

The table below lists some potential problems and possible solutions that may help you troubleshoot your transfection experiments.

Observation	Reason	Solution
Very few or no stably-transfected cells obtained	Improperly cultured cells	• Exactly follow procedures as outlined in **Thawing and Subculturing Cells** (page 15). • Thaw a new batch of early-passage cells. • Do not add antibiotics during transfection.
	Cells not passed 24 hours before transfection	Approximately 24 hours before transfection, pass cells at 3×10^5 cells/mL.
	FreeStyle™ Max Reagent handled incorrectly	• Store at 4°C. **Do not freeze.** • Mix gently by inversion. **Do not vortex.**
	Used poor quality expression construct plasmid DNA (i.e., plasmid DNA from a mini-prep)	**Do not** use miniprep plasmid DNA for transfection. Prepare midiprep plasmid DNA with low endotoxin contamination.
	DNA contaminated	Sterilize DNA using a 0.22 µm filter.
	Used pcDNA™3.3 for single-subunit protein expression	Only clones that are expressed from pOptiVEC™ construct can be selected using HT-deficient CD OptiCHO™ medium and subsequently amplified using MTX. Reclone your gene of interest in pOptiVEC™ TOPO® TA and retransfect your cells.
	Cells experienced too much stress during selection with CD OptiCHO™ medium containing 500 µg/mL of Geneticin® Selective Antibiotic.	Perform two rounds of selection on your transfected cells, one with CD OptiCHO™ medium and one with CD OptiCHO™ medium and 500 µg/mL of Geneticin® Selective Antibiotic.

Continued on next page

Protein Expression

The table below lists some potential problems and possible solutions that may help you troubleshoot your protein expression levels.

Observation	Reason	Solution
No or low protein detected in the supernatant after transient or stable transfection	PCR primer does not contain Kozak translation initiation sequence	Add a Kozak consensus site to the forward PCR primer, resynthesize your DNA and re-clone. See the appropriate manual for each TOPO® TA Cloning Kit for details.
	Premature stop codons	Remove stop codons by your method of choice.
	Improper or ineffective secretion signal	Replace secretion signal. Use endogenous secretion signal if possible.
	Codons not optimized for mammalian cells	Optimize codons for CHO-S cells.

Clone Selection

The table below lists some potential problems and possible solutions that may help you troubleshoot your clone selection experiments.

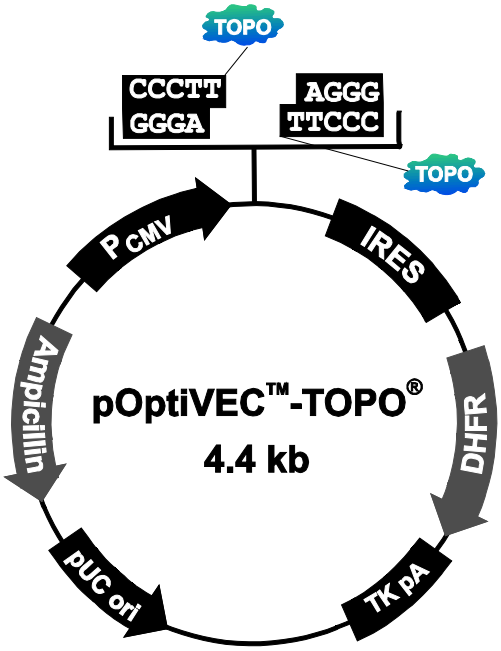
Observation	Reason	Solution
Very few or no single cell clones obtained after cloning	Error in cell counting and dilution during limiting dilution cloning	• Follow procedures exactly as outlined in Cell Counting and Dilution (pages 39 and 59). • Dilute cells no more than 1:3 with a very small amount (< 1mL) of cell suspension.
	Conditioned Medium and HT Supplements omitted from the cloning medium	High cloning efficiency in LDC with CD FortiCHO™ Medium can be achieved with the addition of conditioned media and HT Supplement.
	Conditioned Medium not generated correctly	Follow procedures exactly as outlined in Conditioned Medium Preparation, including the cell seeding density and harvest day.
	Plates moved too soon after seeding the cells	Seeded plates should be incubated undisturbed for 10–14 days.
	Colonies not growing in CD FortiCHO™ Medium	Test CD OptiCHO™ as the cloning medium.

Appendix B: Vectors

Map and features of pOptiVEC™-TOPO® vector

Map

The map below shows the elements of the pOptiVEC™-TOPO® vector. **The vector sequence is available from www.lifetechnologies.com or by contacting Technical Support (page 76).**



Comments for pOptiVEC™-TOPO® 4402 nucleotides

CMV promoter:	36-715
CMV forward primer binding site:	573-593
TOPO® cloning site:	730
EMCV IRES:	772-1359
EMCV IRES reverse primer binding site:	810-829
DHFR:	1372-1935
TK polyadenylation signal:	1975-2247
pUC origin (c):	2609-3282
Ampicillin (*bla*) resistance gene (c):	3424-4284
bla promoter (c):	4279-4383

(c) = complementary strand

Continued on next page

Map and features of pOptiVEC™-TOPO® vector, continued

Features

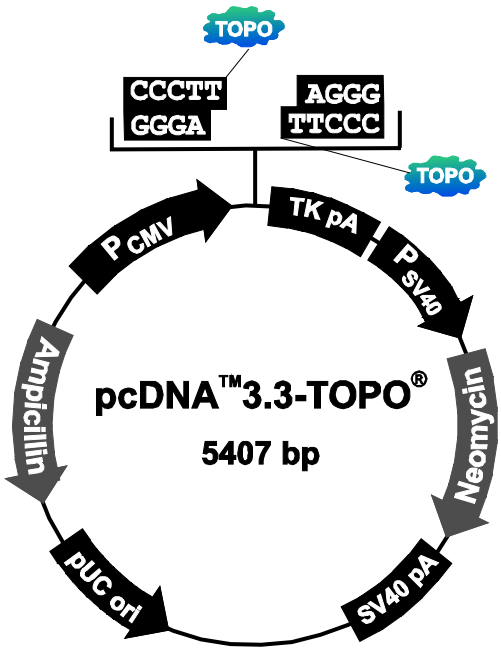
The pOptiVEC™-TOPO® vector contains the following elements. Features have been functionally tested, and the vectors have been fully sequenced.

Feature	Benefit
Full length human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Hennighausen & Fleckenstein, 1986; Nelson *et al.*, 1987).
CMV forward primer	Allows sequencing of the insert.
TOPO® Cloning site	Allows insertion of your PCR product.
EMCV IRES reverse primer	Allows sequencing of the insert.
Internal Ribosome Entry Site (IRES) from the Encephalomyocarditis virus (EMCV)	Allows cap-independent translation of DHFR (Gurtu *et al.*, 1996; Rees *et al.*, 1996).
Dihydrofolate reductase (DHFR) gene	Allows auxotrophic selection of transfected DG44 cells and for genomic amplification of stable cell lines using methotrexate (MTX) (Kaufman *et al.*, 1985).
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Cole & Stacy, 1985).
pUC origin	Allows high-copy number replication and growth in *E. coli.*
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli.*

Map and features of pcDNA™3.3-TOPO® vector

Map

The map below shows the elements of the pcDNA™3.3-TOPO®vector. **The vector sequence is available from www.lifetechnologies.com or by contacting Technical Support (page 76).**



Comments for pcDNA™3.3-TOPO® 5407 nucleotides

CMV promoter:	47-726
CMV forward primer binding site:	584-604
TOPO® cloning site:	741
TK pA reverse primer binding site:	787-805
TK polyadenylation signal:	780-1051
f1 replication origin:	1087-1515
SV40 early promoter:	1520-1889
Neomycin Resistance gene:	1925-2719
SV40 polyadenylation signal:	2895-3025
pUC origin (c):	3408-4081
Ampicillin (*bla*) resistance gene (c):	4226-5086
bla promoter (c):	5087-5185

(c) = complementary strand

Continued on next page

Map and features of pcDNA™3.3-TOPO® vector, continued

Features

The pcDNA™3.3-TOPO® vector contains the following elements. Features have been functionally tested, and the vectors have been fully sequenced.

Feature	Benefit
Full length human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Hennighausen & Fleckenstein, 1986; Nelson *et al.*, 1987).
CMV forward primer	Allows sequencing of the insert.
TOPO® Cloning site	Allows insertion of your PCR product.
TK polyA reverse primer	Allows sequencing of the insert.
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Cole & Stacy, 1985).
SV40 early promoter and origin	Allows efficient, high-level expression of the neomycin resistance gene and episomal replication in cells expressing the SV40 large T antigen.
Neomycin resistance gene	Allows selection of stable transfectants in mammalian cells (Southern & Berg, 1982).
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
pUC origin	Allows high-copy number replication and growth in *E. coli.*
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli.*

Appendix C: Ordering information

Accessory products

Freedom™ DG44 Kit Products

Many of the components supplied with the Freedom™ DG44 Kit are also available separately. Ordering information is provided below. For more information, go to **www.lifetechnologies.com** or contact Technical Support (see page 76).

Item	Amount	Catalog no.
CHO DG44 Cells (cGMP banked) and Media Kit (includes 1×10^7 CHO DG44 Cells, 1000 mL CD DG44 media, L-glutamine, and Pluronic® F-68)	1 kit	A11000-01
pOptiVEC™-TOPO® TA Cloning Kit	1 kit	12744-017
pcDNA™3.3-TOPO® TA Cloning Kit	1 kit	K8300-01
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions 20 reactions	C4040-10 C4040-03
FreeStyle™ MAX Reagent	1 mL	16447-100
OptiPRO™ SFM	100 mL 1000 mL	12309-050 12309-019
L-glutamine, 200 mM, liquid	100 mL	25030-081
L-glutamine, 200 mM, liquid (for EU customers only)	100 mL	25030-024
CD DG44 Medium	1000 mL	12610-010
CD OptiCHO™ Medium	1000 mL	12681-011
Geneticin® Selective Antibiotic, powder	1 g 5 g 25 g	11811-023 11811-031 11811-098
Geneticin® Selective Antibiotic, liquid	20 mL 100 mL	10131-035 10131-027

Gibco® custom media & PD-Direct® services

Through our Gibco® custom media capability and PD-Direct® services, we can develop cloning or growth media formulations specifically suited to your cells. We can provide the best nutrient media delivery scheme for your recombinant cell line, optimizing a Gibco® medium or one in the public domain, or your own formulation. All final media manufacturing is performed in our ISO-9001 certified, QSR/cGMP-compliant facilities and held to the same high standards as our own Gibco® catalog products, ensuring scalability, robustness, and compliance. For more information, go to **www.lifetechnologies.com** or contact Technical Support (see page 76).

Continued on next page

Accessory products, continued

Additional products

The products listed below may be used with the Freedom™ DG44 Kit. For more information, go to **www.lifetechnologies.com** or contact Technical Support (see page 76).

Item	Amount	Catalog no.
FreeStyle™ CHO-S™ Cells	1×10^7 cells	R800-07
FreeStyle™ CHO Expression Medium	1000 mL	12651-014
Pluronic® F-68, 10%	100 mL	24040-032
HT Supplement (100X)	50 mL	11067-030
CD FortiCHO™ Medium	1000 mL	A11483-01
Trypan Blue Solution, 0.4%	100 mL	15250-061
Countess® Automated Cell Counter (includes 50 Countess® cell counting chamber slides and 2 mL of Trypan Blue Stain)	1 unit	C10227
PureLink® HiPure Plasmid Midiprep Kit	25 preps	K2100-04
Anti-Clumping Agent	20 mL	0010057AE
FoamAway™ Irradiated AOF	500 mL in a 1000 mL bag	A10369-02
Neon® Transfection System	1 each	MPK5000

Appendix D: Safety

Chemical safety

WARNING!

GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING!

BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **www.cdc.gov**

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

Appendix E: Purchaser notification

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- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
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Notes